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# Sigma1 receptors inhibit store-operated $\text{Ca}^{2+}$ entry by attenuating coupling of STIM1 to Orai1

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Sigma1 receptors ( $\sigma$ 1Rs) are expressed widely; they bind diverse ligands, including psychotropic drugs and steroids, regulate many ion channels, and are implicated in cancer and addiction. It is not known how  $\sigma$ 1Rs exert such varied effects. We demonstrate that  $\sigma$ 1Rs inhibit store-operated  $\text{Ca}^{2+}$  entry (SOCE), a major  $\text{Ca}^{2+}$  influx pathway, and reduce the  $\text{Ca}^{2+}$  content of the intracellular stores. SOCE was inhibited by expression of  $\sigma$ 1R or an agonist of  $\sigma$ 1R and enhanced by loss of  $\sigma$ 1R or an antagonist. Within the endoplasmic reticulum (ER),  $\sigma$ 1R associated with STIM1, the ER  $\text{Ca}^{2+}$  sensor that regulates SOCE. This interaction was modulated by  $\sigma$ 1R ligands. After depletion of  $\text{Ca}^{2+}$  stores,  $\sigma$ 1R accompanied STIM1 to ER–plasma membrane (PM) junctions where STIM1 stimulated opening of the  $\text{Ca}^{2+}$  channel, Orai1. The association of STIM1 with  $\sigma$ 1R slowed the recruitment of STIM1 to ER–PM junctions and reduced binding of STIM1 to PM Orai1. We conclude that  $\sigma$ 1R attenuates STIM1 coupling to Orai1 and thereby inhibits SOCE.

## Introduction

Sigma1 receptors ( $\sigma$ 1Rs) are widely distributed in the brain and peripheral tissues, including lung, kidney, liver, and spleen, and highly expressed in some tumor cells (Walker et al., 1990; Vilner et al., 1995; Monnet, 2005; Stone et al., 2006; Cobos et al., 2008; Wu and Bowen, 2008; Su et al., 2010; Brune et al., 2013). They are regulated by an unusually diverse array of ligands, including endogenous steroids, drugs of abuse such as cocaine and methamphetamine, and drugs used to treat depression, anxiety, psychosis, pain, and neurodegenerative diseases (Maurice et al., 1999; Waterhouse et al., 2007; Maurice and Su, 2009; Su et al., 2010; Navarro et al., 2012; Robson et al., 2012; Wünsch, 2012; Kourrich et al., 2013; Tsai et al., 2014). Changes in expression and polymorphisms of  $\sigma$ 1Rs are associated with heart failure (Ito et al., 2012, 2013), addiction (Maurice et al., 2002; Kourrich et al., 2013), neurodegenerative and psychiatric disorders (Miki et al., 2014; Tsai et al., 2014), and cancer (Spruce et al., 2004; Wang et al., 2004; Aydar et al., 2006; Maurice and Su, 2009; Crottès et al., 2013). These associations have provoked interest in  $\sigma$ 1Rs as both therapeutic targets and diagnostic tools (van Waarde et al., 2015).

The  $\sigma$ 1R is an integral membrane protein with two trans-membrane domains. It is expressed in the ER, where it is

concentrated in cholesterol-rich mitochondrion-associated ER membrane (MAM) domains and bound to the ER luminal chaperone, BiP (Fig. S1; Hayashi and Su, 2003, 2007; Palmer et al., 2007). Agonists of  $\sigma$ 1R cause it to dissociate from BiP and MAM, allowing  $\sigma$ 1Rs to move within ER membranes and interact with signaling proteins in the plasma membrane (PM), most notably ion channels, thereby regulating their activity (Su et al., 2010; Balasuriya et al., 2012; Pabba, 2013). Antagonists block this effect (Fig. S1 and Table S1). Loss of  $\text{Ca}^{2+}$  from the ER can also release  $\sigma$ 1Rs from their interaction with BiP, freeing them to interact with other proteins (Hayashi and Su, 2007). In addition to regulating the activity of these proteins,  $\sigma$ 1Rs can also act as chaperones, stabilizing signaling proteins as they traffic along the secretory pathway (Tsai et al., 2014).  $\sigma$ 1Rs may also be expressed in the nuclear envelope (Hayashi and Su, 2005a,b; Brune et al., 2013; Mori et al., 2013) and PM (Lupardus et al., 2000; Aydar et al., 2002; Brune et al., 2013; Balasuriya et al., 2014a) and may even be secreted into the extracellular space (Hayashi and Su, 2003; Su et al., 2010). The interactions between  $\sigma$ 1Rs and ion channels may therefore occur within the plane of a membrane (ER or PM) or across ER–PM junctions (Hayashi and Su, 2007; Kourrich et al., 2013; Balasuriya et al., 2014a). Clearly,  $\sigma$ 1Rs are important links between diverse ligands, physiological stimuli, and many key signaling molecules (Hayashi and Su, 2007; Su et al., 2010; Kourrich et al., 2013).

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Abbreviations used in this paper: AFM, atomic force microscopy; ANOVA, analysis of variance; CAD, channel-activating domain; CPA, cyclopiazonic acid; HBS, HEPES-buffered saline; HEK, human embryonic kidney;  $\text{IP}_3$ R,  $\text{IP}_3$  receptor; MAM, mitochondrion-associated ER membrane; NFAT, nuclear factor of activated T cells; PM, plasma membrane; POST, partner of STIM1; SARAF, SOCE-associated regulatory factor;  $\sigma$ 1R, sigma1 receptor; SOCE, store-operated  $\text{Ca}^{2+}$  entry; STIM, stromal interaction molecule; TIRF, total internal reflection fluorescence; TS, Triton solution.

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Receptors that stimulate PLC and formation of inositol 1,4,5-trisphosphate (IP<sub>3</sub>) evoke both Ca<sup>2+</sup> release from the ER through IP<sub>3</sub> receptors (IP<sub>3</sub>Rs) and Ca<sup>2+</sup> entry across the PM. At MAMs, Ca<sup>2+</sup> released by IP<sub>3</sub>Rs can be rapidly accumulated by mitochondria, thereby stimulating oxidative phosphorylation (Rizzuto et al., 2012) and promoting cell survival (Cárdenas et al., 2010), whereas excessive mitochondrial Ca<sup>2+</sup> uptake triggers apoptosis (Mallilankaraman et al., 2012). The association of IP<sub>3</sub>Rs with  $\sigma$ 1Rs at MAMs supports the transfer of Ca<sup>2+</sup> from the ER to mitochondria by curtailing the degradation of active IP<sub>3</sub>Rs (Hayashi and Su, 2007). The increase in mitochondrial Ca<sup>2+</sup> concentration and resultant boost in oxidative phosphorylation are thought to contribute to the prosurvival effects of  $\sigma$ 1Rs in the central nervous system and cancer cells (Lewis et al., 2014). One effect of  $\sigma$ 1Rs may therefore be to support transfer of Ca<sup>2+</sup> from the ER to mitochondria, but this transfer also depends on the Ca<sup>2+</sup> content of the ER.

The Ca<sup>2+</sup> entry evoked by receptors that stimulate PLC is usually mediated by store-operated Ca<sup>2+</sup> entry (SOCE), which is stimulated by loss of Ca<sup>2+</sup> from the ER (Parekh and Putney, 2005; Hogan and Rao, 2015). The reduction in Ca<sup>2+</sup> concentration within the ER is detected by the luminal EF hands of stromal interaction molecule 1 (STIM1), an integral ER membrane protein. This causes STIM1 to cluster and accumulate at ER–PM junctions. STIM1 then binds to Orai1, a Ca<sup>2+</sup>-permeable channel in the PM, and activates it (Lewis, 2007; Soboloff et al., 2012; Wu et al., 2014). The contributions of related proteins (Orai2, Orai3, and STIM2) to SOCE are not fully resolved (Hoth and Niemeyer, 2013), although STIM2 is usually more important than STIM1 for refilling of Ca<sup>2+</sup> stores (Brandman et al., 2007). Additional proteins, including juncate, CRACR2, and SOCE-associated regulatory factor (SARAF), also interact with STIM1–Orai1 signaling complexes and regulate both activation and deactivation of SOCE (Srikanth et al., 2010, 2012, 2013; Palty et al., 2012; Srikanth and Gwack, 2012, 2013).

We show that  $\sigma$ 1Rs constitutively inhibit SOCE and reduce the Ca<sup>2+</sup> content of the ER and that  $\sigma$ 1R ligands modulate this inhibition. The  $\sigma$ 1R associates with STIM1 in the ER and is conveyed with STIM1 to ER–PM junctions after store depletion. This association slows the recruitment of STIM1 to the junctions and reduces binding of STIM1 to Orai1. Our results establish that  $\sigma$ 1Rs inhibit a ubiquitous Ca<sup>2+</sup> entry pathway and suggest a general model for directed translocation of  $\sigma$ 1R to its PM targets.

## Results

### $\sigma$ 1R inhibits SOCE

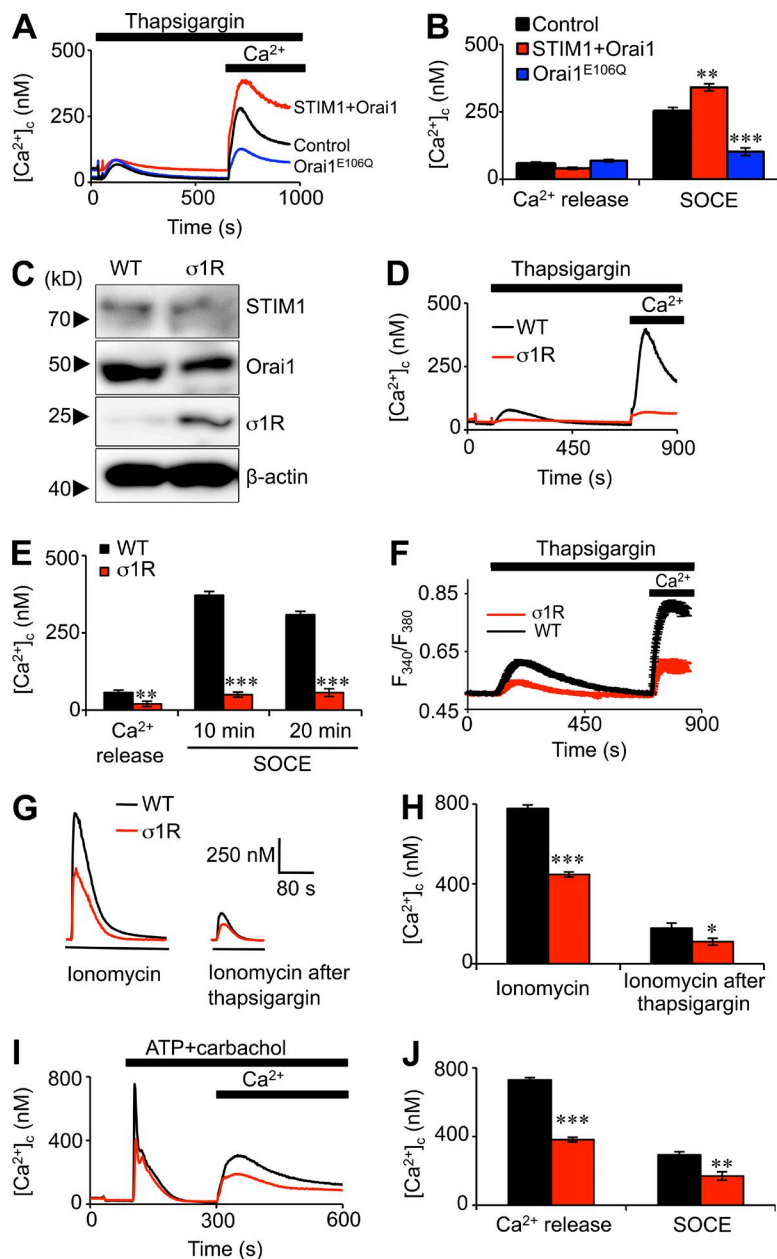
SOCE in human embryonic kidney (HEK) cells can be activated by depletion of intracellular Ca<sup>2+</sup> stores using thapsigargin to inhibit the ER Ca<sup>2+</sup> pump or by stimuli of endogenous receptors (e.g., ATP or carbachol) that activate PLC. The contributions of Orai1 and STIM1 to SOCE (Parekh and Putney, 2005; DeHaven et al., 2009; Soboloff et al., 2012) are clear from the inhibition of thapsigargin-evoked Ca<sup>2+</sup> entry in HEK cells expressing a dominant-negative form of Orai1 (Orai1<sup>E106Q</sup>; Prakriya et al., 2006) and the enhancement of SOCE after overexpression of Orai1 with STIM1 (Fig. 1, A and B). The initial Ca<sup>2+</sup> release evoked by thapsigargin was unaffected by these effects of Orai1 and STIM on SOCE. Stable expression of a V5-tagged  $\sigma$ 1R in HEK cells (HEK- $\sigma$ 1R cells) attenuated the Ca<sup>2+</sup> signals

evoked by thapsigargin without affecting expression of Orai1 or STIM1 ( $103 \pm 5\%$  and  $91 \pm 8\%$  of wild-type cells, respectively; Fig. 1 C) or the basal cytosolic free Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>c</sub>;  $45 \pm 7$  nM and  $50 \pm 3$  nM in wild-type and HEK- $\sigma$ 1R cells, respectively). The increase in [Ca<sup>2+</sup>]<sub>c</sub> after addition of thapsigargin in Ca<sup>2+</sup>-free medium was reduced by  $65 \pm 9\%$ , and the SOCE detected after restoration of extracellular Ca<sup>2+</sup> was reduced by  $86 \pm 4\%$  in HEK- $\sigma$ 1R cells (Fig. 1, D and E). The rate of increase of [Ca<sup>2+</sup>]<sub>c</sub> during SOCE decreased from  $8.8 \pm 0.3$  nM·s<sup>-1</sup> in wild-type HEK cells to  $2.8 \pm 0.3$  nM·s<sup>-1</sup> in HEK- $\sigma$ 1R cells. SOCE in HEK- $\sigma$ 1R cells was similarly reduced across a range of extracellular Ca<sup>2+</sup> concentrations (Fig. S2 A). The inhibition of both thapsigargin-evoked Ca<sup>2+</sup> release and SOCE in HEK- $\sigma$ 1R cells was also observed at 37°C (Fig. S2 B) and in single-cell measurements (Fig. 1 F). The diminished SOCE did not result from ineffective store emptying because it was unaffected by prolonging the incubation with thapsigargin from 10 to 20 min (Fig. 1 E). Indeed, both the initial Ca<sup>2+</sup> content of the stores (determined by addition of ionomycin in Ca<sup>2+</sup>-free medium, the effects of which are not restricted to the ER) and the residual content after thapsigargin treatment were reduced in HEK- $\sigma$ 1R cells (Fig. 1, G and H). When ATP and carbachol were used to deplete Ca<sup>2+</sup> stores via endogenous pathways, the Ca<sup>2+</sup> release and Ca<sup>2+</sup> entry were also attenuated in HEK- $\sigma$ 1R cells (Fig. 1, I and J). The lesser Ca<sup>2+</sup> release evoked by ATP and carbachol in HEK- $\sigma$ 1R cells ( $52 \pm 8\%$  of wild-type cells) matched the reduced Ca<sup>2+</sup> content of the stores ( $59 \pm 9\%$ ), suggesting that this was responsible for the diminished response to PLC-coupled receptors.

To investigate whether sustained depletion of Ca<sup>2+</sup> stores might itself cause down-regulation of SOCE, HEK cells were treated with cyclopiazonic acid (CPA) to reversibly inhibit the ER Ca<sup>2+</sup> pump for a 2-h period that later experiments (see Fig. 3) show is sufficient for  $\sigma$ 1R agonists to inhibit SOCE. This CPA treatment caused a more substantial depletion of the intracellular Ca<sup>2+</sup> stores than was observed in HEK- $\sigma$ 1R cells, but a much smaller inhibition of the SOCE evoked by subsequent addition of thapsigargin (Fig. 2, A and B). These results establish that loss of Ca<sup>2+</sup> from the ER does not cause the reduced SOCE in HEK- $\sigma$ 1R cells.

The smaller increase in [Ca<sup>2+</sup>]<sub>c</sub> evoked by SOCE in HEK- $\sigma$ 1R cells could result from decreased Ca<sup>2+</sup> entry or enhanced Ca<sup>2+</sup> extrusion. However, rates of recovery from Ca<sup>2+</sup> signals evoked by carbachol and ATP in Ca<sup>2+</sup>-free medium (measured over matched [Ca<sup>2+</sup>]<sub>c</sub>) were unaffected by expression of  $\sigma$ 1R (half-times,  $t_{1/2} = 36 \pm 5$  s and  $32 \pm 6$  s for wild-type and HEK- $\sigma$ 1R cells, respectively). The smaller [Ca<sup>2+</sup>]<sub>c</sub> increases in HEK- $\sigma$ 1R cells were not, therefore, due to more effective buffering or Ca<sup>2+</sup> extrusion. Because most Ca<sup>2+</sup> extrusion pathways do not transport Mn<sup>2+</sup>, we used quenching of fura 2 fluorescence to measure unidirectional Mn<sup>2+</sup> influx through the SOCE pathway (Fig. 2 C). Thapsigargin, or carbachol with ATP, stimulated Mn<sup>2+</sup> entry in HEK cells, consistent with the activation of SOCE in response to store depletion. In HEK- $\sigma$ 1R cells, there was no change in the rate of Mn<sup>2+</sup> entry in response to either stimulus (Fig. 2 D). Together, these results establish that stable expression of  $\sigma$ 1R inhibits SOCE.

Selection of polyclonal HEK cells stably expressing  $\sigma$ 1R might have propagated cells with different Ca<sup>2+</sup> signaling behaviors. However, the thapsigargin-evoked increase in [Ca<sup>2+</sup>]<sub>c</sub> and SOCE and the Ca<sup>2+</sup> content of the intracellular stores were also reduced in HEK cells transiently expressing  $\sigma$ 1Rs (Fig. S2,



**Figure 1. Inhibition of SOCE by  $\sigma$ 1R.** (A)  $\text{Ca}^{2+}$  signals recorded from populations of fluo 4-loaded HEK cells transiently transfected with Orai1<sup>E106Q</sup>, STIM1 and Orai1, or mock transfected (control). Cells were stimulated with 5  $\mu\text{M}$  thapsigargin in  $\text{Ca}^{2+}$ -free HBS before restoration of extracellular  $\text{Ca}^{2+}$  (final free  $[\text{Ca}^{2+}]$ , 4 mM). Results show mean responses from six replicates. (B) Summary results ( $n = 3$ ) show peak increases in  $[\text{Ca}^{2+}]_i$  evoked by thapsigargin ( $\text{Ca}^{2+}$  release) and  $\text{Ca}^{2+}$  restoration (SOCE). (C) Typical immunoblot of  $\sigma$ 1R, STIM1, Orai1, and  $\beta$ -actin from 20  $\mu\text{g}$  of solubilized protein from wild-type (WT) HEK and HEK- $\sigma$ 1R cells. (D)  $\text{Ca}^{2+}$  signals evoked by thapsigargin in  $\text{Ca}^{2+}$ -free HBS and after restoration of extracellular  $\text{Ca}^{2+}$  to wild-type and HEK- $\sigma$ 1R cells. (E) Summary shows responses to thapsigargin ( $\text{Ca}^{2+}$  release) and SOCE detected after restoring  $\text{Ca}^{2+}$  10 or 20 min after thapsigargin ( $n = 6$ ). (F) Responses from single fura 2-loaded HEK cells show fluorescence ratios ( $F_{340}/F_{380}$ ) after stimulation with 5  $\mu\text{M}$  thapsigargin and restoration of 4 mM extracellular  $\text{Ca}^{2+}$ .  $n = 3$ , each with  $\sim 45$  cells. (G)  $\text{Ca}^{2+}$  contents of the intracellular stores determined by measuring  $[\text{Ca}^{2+}]_i$  after addition of 5  $\mu\text{M}$  ionomycin in  $\text{Ca}^{2+}$ -free HBS before or 10 min after treatment with thapsigargin. (H) Summary results ( $n = 6$ ). (I)  $\text{Ca}^{2+}$  release and SOCE evoked by 100  $\mu\text{M}$  carbachol and 100  $\mu\text{M}$  ATP. (J) Summary results ( $n = 6$ ). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . Student's  $t$  test (E, H, and J) or ANOVA with Tukey's posthoc analysis (B). Results show mean  $\pm$  SEM.

C–E). The reduced SOCE correlated with the level of expression of  $\sigma$ 1R (Fig. S2, F and G). Translocation of GFP-tagged nuclear factor of activated T cells (NFAT) from the cytosol to the nucleus requires SOCE (Kar et al., 2011). SOCE stimulated NFAT translocation in HEK cells, and the response was attenuated to similar degrees in cells stably or transiently expressing  $\sigma$ 1R (Fig. 2, E and F).

We conclude that expression of  $\sigma$ 1R inhibits SOCE by reducing the coupling of empty stores to the activation of SOCE.

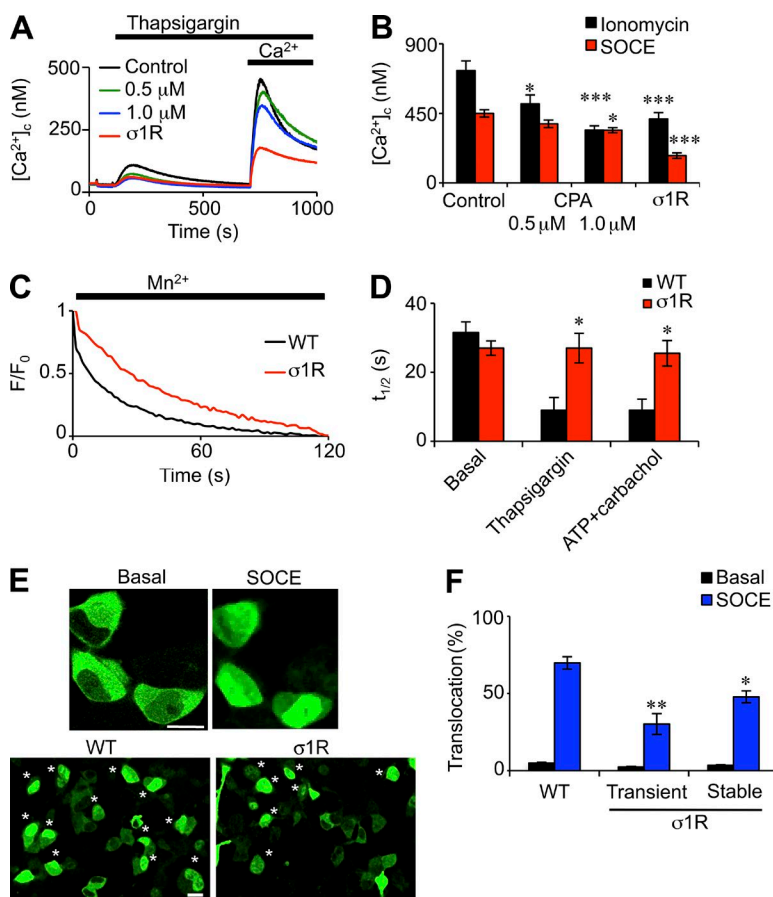
#### Agonists and antagonists of $\sigma$ 1R regulate SOCE

The  $\sigma$ 1R agonist (+)SKF10047 (Su et al., 2010; Navarro et al., 2012) and the antagonist BD1047 (Fig. S1; Skuza and Rogó, 2006; Gromek et al., 2014) were used to investigate the acute effects of  $\sigma$ 1Rs in CHO cells and HEK- $\sigma$ 1R cells. In CHO cells,  $\sigma$ 1Rs are endogenously expressed (Hayashi and Su, 2007). As in HEK cells, SOCE was inhibited by transient expression of

Orai1<sup>E106Q</sup>, although in CHO cells, the thapsigargin-evoked  $\text{Ca}^{2+}$  release was also inhibited (Fig. S3 A). In both CHO and HEK- $\sigma$ 1R cells, preincubation with BD1047 increased the amplitude of the  $\text{Ca}^{2+}$  signals evoked by SOCE, whereas the agonist (+)SKF10047 had the opposite effect (Fig. 3, A–D). Neither ligand affected SOCE in wild-type HEK cells (Fig. 3, E and F), confirming that the effects are mediated by  $\sigma$ 1Rs. The temperature dependence and slow equilibration of ligand binding to  $\sigma$ 1Rs (Yamamoto et al., 2001; Chu and Ruoho, 2016), together with the need to load cells with  $\text{Ca}^{2+}$  indicators at 20°C, limited opportunities to investigate the time course of the effects of  $\sigma$ 1R ligands. Nevertheless, it is clear that treatment with ligands for at least 1 h at 37°C before loading cells with  $\text{Ca}^{2+}$  indicator (1.5 h) in the continued presence of ligands was required to detect significant effects of the ligands on SOCE (Fig. S3, B–D).

In CHO cells, siRNA to  $\sigma$ 1R almost abolished expression of endogenous  $\sigma$ 1R, but this was accompanied by reduced expression of Orai1 and increased expression of STIM1 (Fig. 3, G





**Figure 2. Stable and transient expression of  $\sigma$ 1R inhibits SOCE.** (A)  $Ca^{2+}$  signals evoked by 1  $\mu$ M thapsigargin in  $Ca^{2+}$ -free HBS followed by restoration of 4 mM extracellular  $Ca^{2+}$  in HEK wild-type cells treated with CPA (0.5  $\mu$ M or 1  $\mu$ M for 2.5 h) or HEK- $\sigma$ 1R cells. (B) Summary results show peak increases in  $[Ca^{2+}]_i$  evoked by SOCE or by addition of ionomycin in  $Ca^{2+}$ -free HBS ( $n = 3$ ). (C) Populations of fura 2-loaded cells were treated with thapsigargin (5  $\mu$ M for 10 min) in nominally  $Ca^{2+}$ -free HBS before addition of 5 mM  $MnCl_2$ . Results show normalized fluorescence intensity ( $F/F_0$ ) for six replicates. WT, wild type. (D) Summary results ( $n = 3$ ) show half-times ( $t_{1/2}$ ) for fluorescence quenching from unstimulated cells (basal) and cells treated with thapsigargin (5  $\mu$ M for 10 min) or ATP and carbachol (100  $\mu$ M each for 3.5 min). (E) Typical images of HEK cells expressing NFAT-GFP before and 30 min after addition of 5  $\mu$ M thapsigargin in normal HBS (top). Bar, 10  $\mu$ m. Images of larger fields (bottom) show thapsigargin-treated HEK wild-type and HEK- $\sigma$ 1R cells. Asterisks indicate cells used for analysis. Bar, 20  $\mu$ m. (F) Summary results show nuclear translocation of NFAT-GFP before and after treatment with thapsigargin (percentage of cells; six independent fields, with between 595 and 660 cells counted for each condition). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . Student's  $t$  test (D) or ANOVA with Tukey's posthoc analysis (B and F). Results show mean  $\pm$  SEM.

and H). The loss of Orai1 could reflect a chaperone role for  $\sigma$ 1R similar to the stabilization of human ether-a-go-go-related gene (HERG)  $K^+$  channels by  $\sigma$ 1R (Hayashi and Su, 2007; Crottès et al., 2011). Alternatively, Orai1 expression may be down-regulated through an adaptive feedback mechanism arising from the reduced inhibition of SOCE after loss of  $\sigma$ 1Rs. Overstimulation of SOCE by constitutively active STIM1 was shown previously to reduce Orai1 expression (Kilch et al., 2013). Despite the reduced expression of Orai1, SOCE was increased in CHO cells lacking  $\sigma$ 1Rs, and it was then unaffected by (+)SKF10047 or BD1047 (Fig. 3, I and J). The enhanced SOCE in CHO cells lacking  $\sigma$ 1Rs was abolished by expression of the dominant-negative form of Orai1 (Fig. S3 G), confirming that it was mediated by Orai1. SOCE monitored by unidirectional  $Mn^{2+}$  entry was also increased in CHO cells treated with siRNA to  $\sigma$ 1R (Fig. S3, E and F). In normal CHO cells, (+)SKF10047 reduced the  $Ca^{2+}$  content of the stores, whereas the  $\sigma$ 1R antagonist BD1047 increased their content to a level that matched that of cells without  $\sigma$ 1Rs. Neither ligand affected the  $Ca^{2+}$  stores in CHO cells lacking  $\sigma$ 1Rs (Fig. 3, K and L). Although comparison of SOCE-mediated  $Ca^{2+}$  signals in CHO cells with and without  $\sigma$ 1Rs is compromised by accompanying changes in STIM1 and Orai1 expression (Fig. 3, G and H), the analyses demonstrate that  $\sigma$ 1R ligands are effective only in cells expressing  $\sigma$ 1Rs, and they establish a constitutive inhibition of SOCE by endogenous  $\sigma$ 1Rs and an associated reduction in ER  $Ca^{2+}$  content in CHO cells (Fig. 3, I–L). Similar results were obtained in HEK- $\sigma$ 1R cells: siRNA to  $\sigma$ 1R abolished the effects of  $\sigma$ 1R ligands on both SOCE and the  $Ca^{2+}$  content of the stores; it also increased the basal  $Ca^{2+}$  content of the stores and the rate of

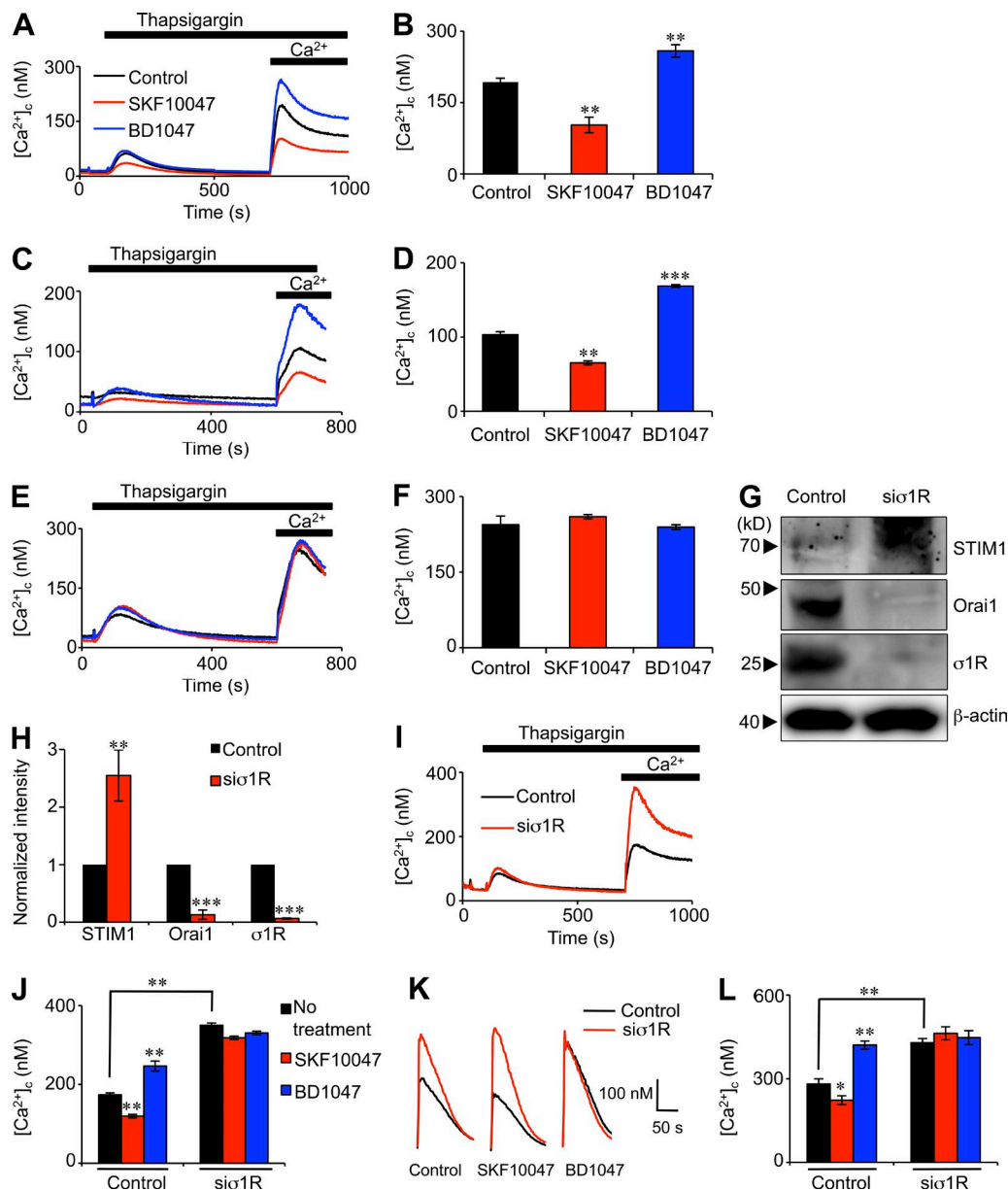
$Mn^{2+}$  entry evoked by either thapsigargin or by the more physiological stimuli, ATP and carbachol (Fig. S3, H and J).

Determining whether ligands of  $\sigma$ 1R are more effective before or after depletion of  $Ca^{2+}$  stores was frustrated by the need for prolonged preincubations at 37°C for optimal effects (Fig. S3, B–D). In a modified protocol, fluo 4-loaded HEK- $\sigma$ 1R cells in  $Ca^{2+}$ -free Hepes-buffered saline (HBS) were incubated with (+)SKF10047 or BD1047 for 2 h at 20°C, with thapsigargin added either immediately before the ligands or after the 2-h incubation. Under these conditions, where the effects of the ligands were much reduced, (+)SKF10047 modestly inhibited SOCE, and BD1047 modestly enhanced SOCE, but only when added before store depletion (Fig. S3 K). These results suggest that  $\sigma$ 1R ligands affect an early step in the activation of SOCE.

Breast cancer cells express high levels of  $\sigma$ 1Rs (Spruce et al., 2004; Wang et al., 2004; Aydar et al., 2006). In MDA-MB-231 human breast cancer cells, which also express  $\sigma$ 1Rs, SOCE was enhanced by BD1047 and inhibited by (+)SKF10047 (Fig. S4). The  $Ca^{2+}$  content of the stores was also reduced by (+)SKF10047. Hence, in three cell types, HEK- $\sigma$ 1R, CHO, and MDA-MB-231 cells,  $\sigma$ 1Rs both inhibit SOCE and decrease the  $Ca^{2+}$  content of the ER. The inverse agonist effect of BD1047 in CHO and MDA-MB-231 cells suggests a constitutive regulation of SOCE and ER  $Ca^{2+}$  content by endogenous  $\sigma$ 1Rs.

#### **$\sigma$ 1R and STIM1 associate and move to ER-PM junctions after store depletion**

Interactions between  $\sigma$ 1R and STIM1 in unstimulated cells were investigated using HEK cells transiently expressing STIM1-Myc and  $\sigma$ 1R-FLAG. Anti-Myc beads pulled down  $\sigma$ 1R-FLAG

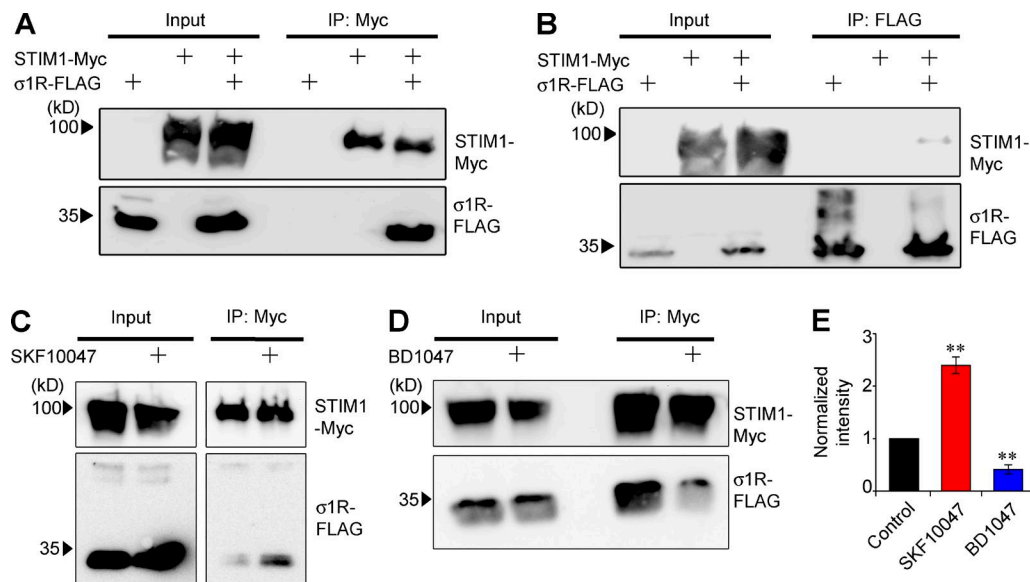


**Figure 3. Ligands of  $\sigma 1R$  modulate SOCE.** (A–F) Populations of cells were treated with 25  $\mu M$  (+)SKF10047 or 10  $\mu M$  BD1047 before removal of extracellular  $Ca^{2+}$ , addition of 5  $\mu M$  thapsigargin, and then restoration of extracellular 4 mM  $Ca^{2+}$  to CHO (A and B), HEK- $\sigma 1R$  (C and D), or wild-type HEK cells (E and F). Summary results (B, D, and F) show peak increases in  $[Ca^{2+}]_c$  after restoration of extracellular  $Ca^{2+}$ . The color codes in A apply to all panels (A–F). (G) Representative immunoblot from CHO cells transfected with control plasmid or plasmid encoding siRNA for  $\sigma 1R$  (si $\sigma 1R$ ). (H) Summary results show band intensities for the indicated proteins normalized to those from cells treated with control plasmid. (I)  $Ca^{2+}$  signals evoked by addition of thapsigargin in  $Ca^{2+}$ -free HBS and then restoration of extracellular  $Ca^{2+}$  in CHO cells treated with si $\sigma 1R$  or control plasmid. (J) Summary shows peak  $[Ca^{2+}]_c$  after restoration of extracellular  $Ca^{2+}$  to thapsigargin-treated CHO cells treated with si $\sigma 1R$  or control plasmid. Cells were pretreated with 25  $\mu M$  (+) SKF10047 or 10  $\mu M$  BD1047, as indicated. (K and L) Effects of si $\sigma 1R$  or control plasmid and pretreatment with  $\sigma 1R$  ligands on the  $Ca^{2+}$  signals evoked by 5  $\mu M$  ionomycin in  $Ca^{2+}$ -free HBS. Typical traces (K) and summary results (L) are shown. Legends for L are the same as J. All summary results show mean  $\pm$  SEM.  $n = 3$ . \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . ANOVA with Tukey's posthoc analysis (B, D, F, J, and L) or Student's  $t$  test (H and comparison of no treatment conditions in J and L).

from solubilized cell extracts, but only in cells expressing STIM1-Myc. Conversely, anti-FLAG beads pulled down STIM1-Myc, but only in cells expressing  $\sigma 1R$ -FLAG (Fig. 4, A and B). Coimmunoprecipitation of  $\sigma 1R$ -FLAG with STIM1-Myc was enhanced by (+)SKF10047 and reduced by BD1047 (Fig. 4, C–E). These results show that STIM1 and  $\sigma 1R$  are associated in unstimulated cells and that their interaction is regulated by  $\sigma 1R$  ligands. Furthermore, the increased association

of  $\sigma 1R$  with STIM1 evoked by the  $\sigma 1R$  agonist (Fig. 4) correlates with the inhibition of SOCE (Fig. 3).

To investigate the intracellular dynamics of  $\sigma 1R$  and STIM1, we used HeLa cells because they are better suited than HEK cells for optical analyses of ER proteins while still lacking detectable endogenous  $\sigma 1R$ s (Fig. 5 A). In cells expressing  $\sigma 1R$ -EGFP with mCh-STIM1,  $\sigma 1R$ -EGFP and mCh-STIM1 colocalized within the ER (Mander's correlation coefficient



**Figure 4. Ligands regulate association of  $\sigma$ 1R with STIM1.** (A and B) Solubilized HEK cells expressing STIM1-Myc,  $\sigma$ 1R-FLAG, or both were immunoprecipitated (IP) with anti-Myc (A) or anti-FLAG (B) antibodies before immunoblotting. Input lanes were loaded with 10  $\mu$ l of the 500- $\mu$ l sample and immunoprecipitation lanes with 20  $\mu$ l of the 50- $\mu$ l eluate. (C and D) Similar immunoprecipitation analyses from cells expressing STIM1-Myc and  $\sigma$ 1R-FLAG after pretreatment with 25  $\mu$ M (+)SKFI0047 or 10  $\mu$ M BD1047. (E) Summary results (normalized to control cells; mean  $\pm$  SEM;  $n = 3$ ) show amounts of  $\sigma$ 1R-FLAG immunoprecipitated by anti-Myc antibody in cells transiently expressing STIM1-Myc and  $\sigma$ 1R-FLAG. \*\*,  $P < 0.01$ . ANOVA with Tukey's posthoc analysis.

was  $0.77 \pm 0.03$ ;  $n = 8$ ; Fig. 5 B). We used total internal reflection fluorescence (TIRF) microscopy to visualize translocation of mCh-STIM1 and  $\sigma$ 1R-EGFP in response to thapsigargin. In cells expressing mCh-STIM1, thapsigargin stimulated an accumulation of mCh-STIM1 in puncta immediately beneath the PM (Fig. 5 C, top). This is consistent with evidence that store depletion causes STIM1 to aggregate into sub-PM clusters, where they interact with Orai1 to activate SOCE (Liou et al., 2007; Wu et al., 2014). In contrast, thapsigargin had no detectable effect on the sub-PM distribution of  $\sigma$ 1R-EGFP expressed alone (Fig. 5 C, bottom). However, when mCh-STIM1 and  $\sigma$ 1R-EGFP were coexpressed, thapsigargin caused both proteins to accumulate in sub-PM puncta, within which the proteins colocalized (Mander's correlation coefficient was  $0.77 \pm 0.04$ ;  $n = 8$ ; Fig. 5 D), but expression of  $\sigma$ 1R slowed the rate of formation of the mCh-STIM1 puncta (Fig. 5 E). Rates of formation of mCh-STIM1 puncta were unaffected by expression of another ER membrane protein, IP<sub>3</sub>R1 (times to 50% accumulation were  $325 \pm 14$  s and  $342 \pm 11$  s, with and without IP<sub>3</sub>R1, respectively), confirming that the effects of  $\sigma$ 1R were not caused by nonspecific accumulation of ER proteins. Furthermore, in cells coexpressing Orai1-EGFP,  $\sigma$ 1R-mKate, and HA-STIM1, Orai1-EGFP and  $\sigma$ 1R-mKate accumulated into colocalized puncta after thapsigargin treatment, but neither formed puncta in the absence of STIM1 (Fig. 5, F and G). These results demonstrate that after store depletion,  $\sigma$ 1R accompanies STIM1 to ER-PM junctions, but  $\sigma$ 1R slows the accumulation of STIM1.

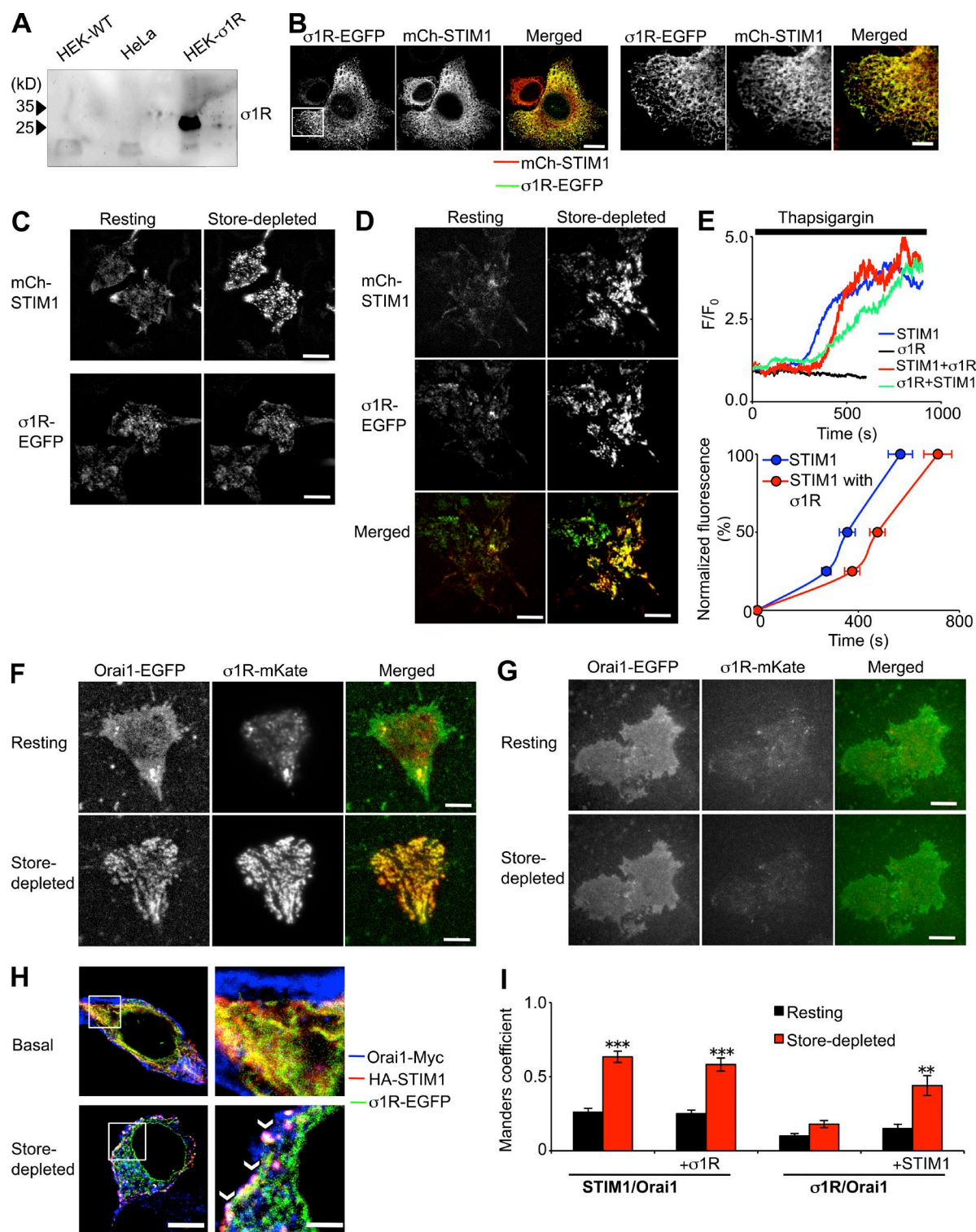
In related experiments, HeLa cells expressing different combinations of  $\sigma$ 1R-EGFP, mCh-STIM1, and Orai1-Myc were fixed for immunolabeling, and confocal images were analyzed to assess colocalization of the proteins before and after treatment with thapsigargin. As expected, in cells coexpressing STIM1 and Orai1, thapsigargin caused their colocalization to increase, consistent with evidence that clustered STIM1 at ER-PM junctions captures Orai1 as it diffuses within the PM (Wu et al., 2014). In contrast, when  $\sigma$ 1R and Orai1 were coexpressed,

their colocalization was enhanced by store depletion only in the presence of STIM1, and overlapping puncta of all three proteins were then apparent at the cell periphery (Fig. 5, H and I). These results agree with those obtained using TIRF microscopy and demonstrate the importance of STIM1 in recruiting both Orai1 and  $\sigma$ 1R to the same junctions.

#### $\sigma$ 1R reduces the association of STIM1 with PM Orai1

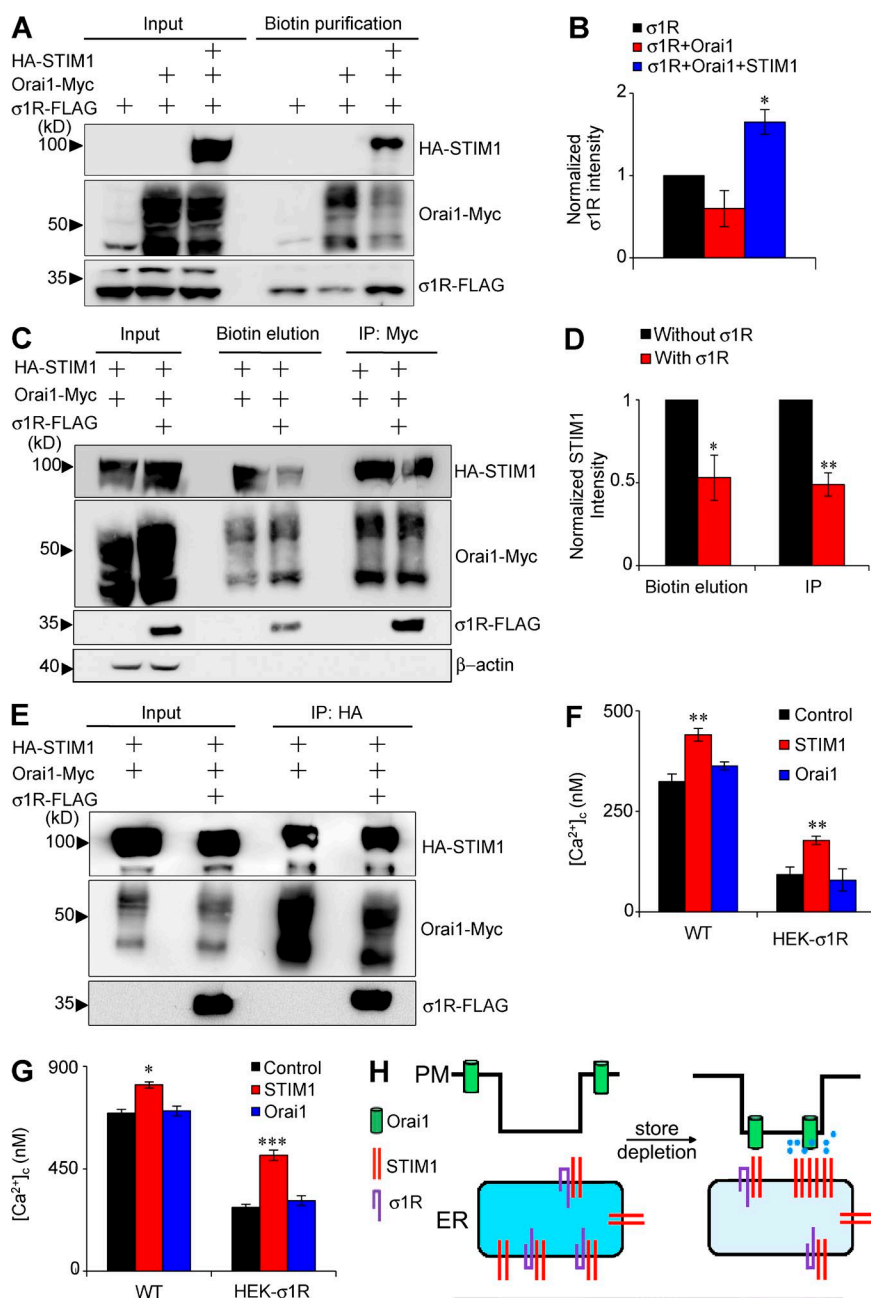
The requirement for STIM1 in recruiting  $\sigma$ 1R to ER-PM junctions containing Orai1 was investigated further by expressing  $\sigma$ 1R-FLAG and Orai1-Myc with and without HA-STIM1. After treatment with thapsigargin and cell surface biotinylation, PM protein complexes were purified using avidin. Immunoblotting showed that the amount of  $\sigma$ 1R within the biotinylated sample was significantly increased in cells overexpressing STIM1 and Orai1, but not when only Orai1 was overexpressed (Fig. 6, A and B). This indicates that STIM1 either promotes trafficking of  $\sigma$ 1R to the PM, where it is directly biotinylated, or it promotes association of  $\sigma$ 1R with a biotinylated PM protein. Similar analyses established that expression of  $\sigma$ 1R-FLAG reduced the amount of STIM1 in the biotinylated sample to  $47 \pm 12\%$  ( $n = 3$ ) of that measured without  $\sigma$ 1R (Fig. 6, C and D). The  $\beta$ -actin control showed no evidence of cell permeabilization or biotinylation of intracellular proteins. The biotinylated PM sample was subjected to a further round of purification using anti-Myc beads. Immunoblotting confirmed that when all three proteins were expressed, they were each captured in the final extract, suggesting that both STIM1 and  $\sigma$ 1R are associated with the PM Orai1 channel complex (and that there is no need to invoke cell surface expression of  $\sigma$ 1R to account for its presence in the biotinylated sample). The amount of STIM1 within this complex was again reduced by  $\sigma$ 1R to  $51 \pm 7\%$  of that measured without  $\sigma$ 1R (Fig. 6, C and D). These results indicate that  $\sigma$ 1R reduces the amount of STIM1 bound to PM Orai1. This was confirmed by purifying HA-STIM1 with anti-HA beads:





**Figure 5.  $\sigma$ 1R accompanies STIM1 to ER-PM junctions after store depletion.** (A) Immunoblot of lysates from wild-type (WT) HEK, HEK- $\sigma$ 1R, or HeLa cells. The same amount of protein was loaded in each lane. (B) Confocal images of unstimulated HeLa cells transiently transfected with  $\sigma$ 1R-EGFP and mCh-STIM1. Bar, 10  $\mu$ m. (Right) Enlargement of the boxed area. Bar, 2.5  $\mu$ m. (C and D) TIRF images of HeLa cells expressing mCh-STIM1 (C, top),  $\sigma$ 1R-EGFP (C, bottom), or both (D) before and 10 min after addition of 5  $\mu$ M thapsigargin in  $\text{Ca}^{2+}$ -free HBS. (E, top) Traces show time courses of the fluorescence changes ( $F/F_0$ ) within the TIRF field after addition of thapsigargin (mean values for 30 puncta for each or size-matched regions of interest for  $\sigma$ 1R alone). (E, bottom) Summary results show changes in mCh fluorescence (normalized to maximal intensity) after store depletion in cells with and without  $\sigma$ 1R ( $n = 87$ ). (F and G) TIRF images of HeLa cells expressing Orai1-EGFP and  $\sigma$ 1R-mKate either with (F) or without HA-STIM1 (G). Bars (C, D, F, and G), 10  $\mu$ m. (H) Confocal images of HeLa cells expressing  $\sigma$ 1R-EGFP, HA-STIM1, and Orai1-Myc, immunostained after treatment with 5  $\mu$ M thapsigargin. Boxed areas in the left panels (bar, 5  $\mu$ m) are enlarged on the right (bar, 2  $\mu$ m). Arrowheads show colocalization of all three proteins as white puncta at the PM. (I) Summary results ( $n = 8$ ) show Mander's overlap coefficient for colocalization of the indicated pairs of proteins in cells expressing only those tagged proteins or with  $\sigma$ 1R-EGFP or HA-STIM1, as indicated, with and without thapsigargin treatment. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ , relative to resting cells. Student's  $t$  test. Results show mean  $\pm$  SEM.





**Figure 6. STIM1, Orai1, and σ1R interact within a macromolecular complex at the PM.** (A) HEK cells expressing σ1R-FLAG alone or with Orai1-Myc or Orai1-Myc and HA-STIM1 were treated with thapsigargin (5 μM for 30 min in Ca<sup>2+</sup>-free HBS), and then the cell surface was biotinylated. The representative immunoblot shows the inputs and the proteins detected after purification with avidin beads. Input lanes were loaded with 10 μl of the 500-μl sample, and surface biotinylation lanes were loaded with 10 μl of the 50-μl eluate. (B) Summary shows the amounts of σ1R-FLAG detected in the avidin pull-downs (normalized to cells expressing only σ1R-FLAG). (C) HEK cells expressing Orai1-Myc and HA-STIM1 with or without σ1R-FLAG were cell surface biotinylated before sequential purification by elution from avidin-agarose with biotin and then from anti-Myc-agarose with Myc peptide. The immunoblot (anti-HA, anti-FLAG, anti-Myc, and anti-β-actin) shows the input and the two eluates. Input lanes were loaded with 10 μl of the 500-μl sample and elution lanes with 10 μl of the 50-μl eluate. (D) Summary shows the amounts of HA-STIM1 detected in the avidin (biotin elution) and anti-Myc pull-downs (normalized to Orai1-Myc pull-down in each condition). (E) HEK cells expressing Orai1-Myc and HA-STIM1 with or without σ1R-FLAG were immunoprecipitated (IP) with anti-HA antibody. (F) Peak [Ca<sup>2+</sup>]<sub>i</sub> signals evoked by SOCE were recorded from HEK or HEK-σ1R cells after treatment with thapsigargin (5 μM in Ca<sup>2+</sup>-free HBS for 10 min) and then restoration of 4 mM extracellular Ca<sup>2+</sup>. The effects of transiently overexpressing STIM1 or Orai1 are shown. WT, wild type. (G) The Ca<sup>2+</sup> contents of the intracellular stores of the same cells were measured by recording peak increases in [Ca<sup>2+</sup>]<sub>i</sub> from cells exposed to ionomycin (5 μM in Ca<sup>2+</sup>-free HBS). Results (B, D, F, and G) are mean ± SEM. *n* = 3. \*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001, relative to control. ANOVA with Tukey's posthoc analysis (B, F, and G) and Student's *t* test (D). (H) The results suggest that STIM1 and σ1R within the ER are associated. When STIM1 is activated by depletion of the ER Ca<sup>2+</sup> stores, STIM1 conveys σ1R to the PM, where STIM1 and Orai1 associate, trapping them within ER-PM junctions. The interaction between STIM1 and Orai1 is weakened by σ1R.

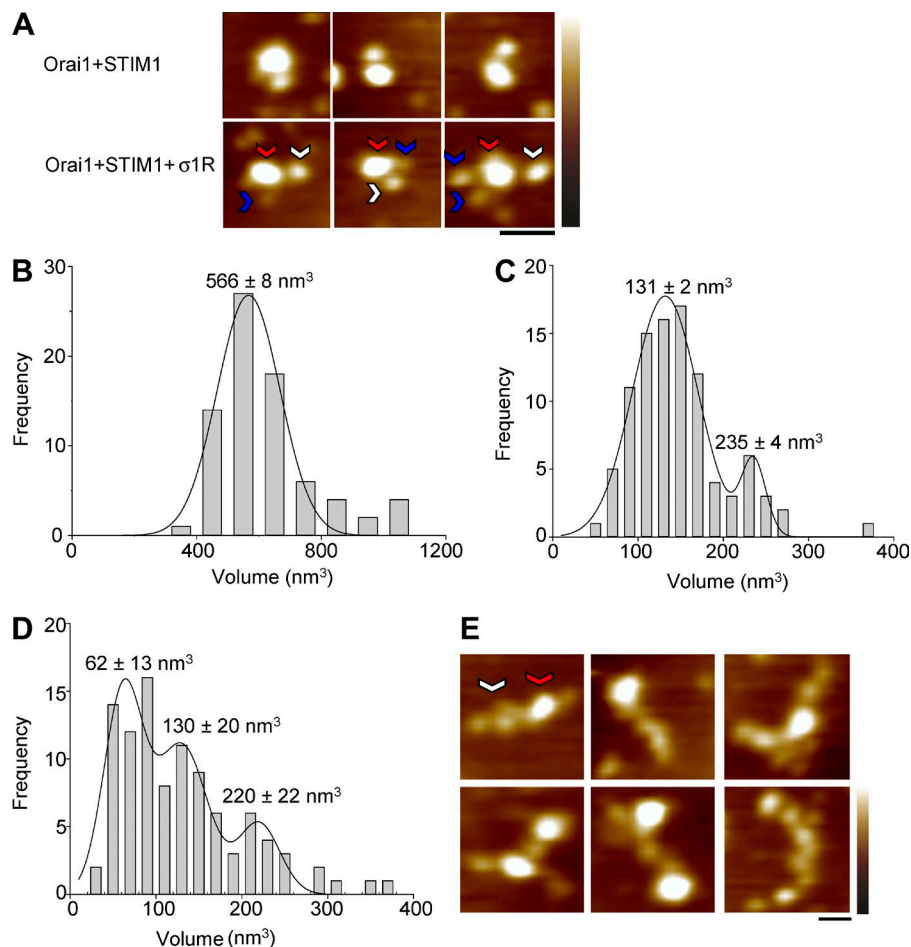
the amount of Orai1 that copurified with STIM1 was reduced in the presence of σ1R (Fig. 6 E).

If the reduction in STIM1 binding to Orai1 contributes to inhibition of SOCE by σ1R, we might expect increased expression of STIM1 to relieve the inhibition. We therefore tested the effects of overexpressing STIM1 on the amplitude of SOCE in wild-type HEK and HEK-σ1R cells. Expression of STIM1 produced a similar increase in the amplitude of SOCE in wild-type and HEK-σ1R cells, but the percent increase was greater in the HEK-σ1R cells (36 ± 5% in wild-type and 81 ± 8% in HEK-σ1R cells; Fig. 6 F). This suggests that activation of SOCE is more limited by STIM1 in HEK-σ1R cells than in wild-type cells. The effects of STIM1 on SOCE were matched by its effects on Ca<sup>2+</sup> stores: overexpression of STIM1 increased the Ca<sup>2+</sup> content of the stores, and the effect was greater in HEK-σ1R relative to wild-type cells (83 ± 7% and 18 ± 5% increases, respectively; Fig. 6 G). Furthermore, the effects of

σ1R ligands on SOCE were much reduced in HEK-σ1R cells overexpressing STIM1 and Orai1 (Fig. S5). These results support the idea that σ1Rs inhibit the association of STIM1 with PM Orai1, thereby reducing SOCE (Fig. 6 H). Coincident with this inhibition of SOCE by σ1Rs, we invariably detected a decrease in the Ca<sup>2+</sup> content of the ER.

#### σ1R inhibits binding of STIM1 to PM Orai1 channel complexes

To examine the structure of the PM Orai1 channel complex in the presence and absence of σ1R, we used atomic force microscopy (AFM). Previous AFM images of complexes purified from cells overexpressing Orai1 and STIM1 showed a hexameric arrangement of STIM1 around a central Orai1 complex and a few strings of STIM1 molecules associated with Orai1, consistent with the oligomerization of STIM1 after depletion of Ca<sup>2+</sup> stores (Balasuriya et al., 2014b). We examined extracts from



**Figure 7. AFM analyses of interactions between  $\sigma$ 1R, STIM1, and Orai1 at the PM.** (A) AFM images of Orai1 isolated with  $\sigma$ 1R and STIM1 showing the central Orai1 (red arrowheads) channel decorated by either STIM1 (white arrowheads) or both STIM1 and  $\sigma$ 1R (blue arrowheads). (B–D) Frequency distributions of the volumes of the decorated central particles (Orai1; B), bound peripheral particles (STIM1) from cells expressing Orai1 and STIM1 (C), and bound peripheral particles ( $\sigma$ 1R and STIM1) from cells expressing Orai1, STIM1, and  $\sigma$ 1R (D). For these analyses, volume ranges of 30–100 nm<sup>3</sup> and 120–300 nm<sup>3</sup> were set for  $\sigma$ 1R and STIM1 particles, respectively; the intermediate volumes (100–120 nm<sup>3</sup>) were disregarded. (E) AFM images showing strings of STIM1 (white arrowhead) connecting several Orai1 channels (red arrowhead). These infrequent structures were observed only in the absence of  $\sigma$ 1Rs. Bars, 20 nm; height scales, 0–3 nm (dark to light).

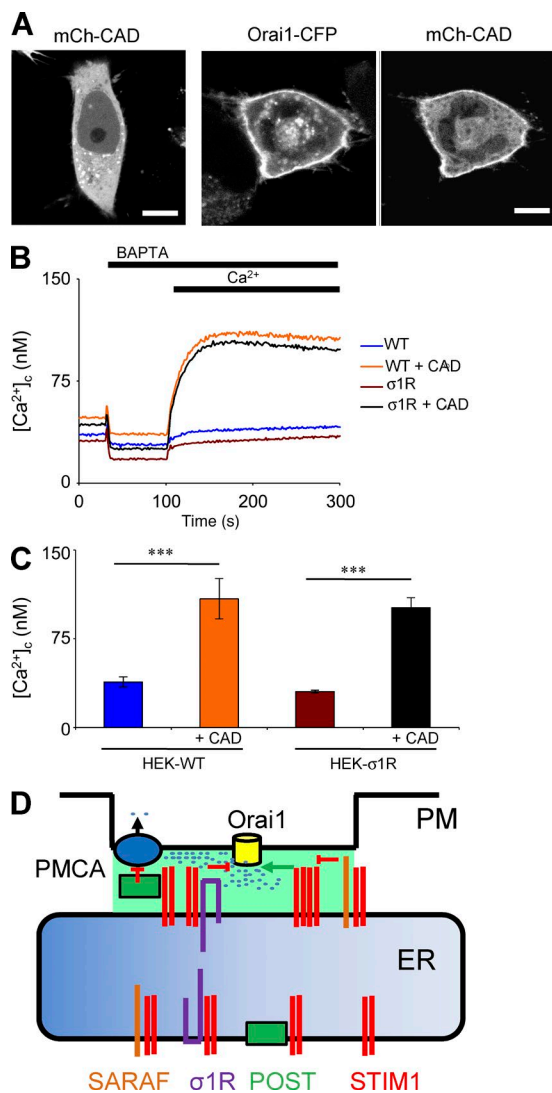
thapsigargin-treated HEK cells expressing Orai1-Myc/His and HA-STIM1, with or without  $\sigma$ 1R-FLAG, in which cell surface proteins had been biotinylated and complexes were isolated by sequential purification using avidin and anti-Myc beads. AFM images showed large particles decorated by smaller peripheral particles (Fig. 7 A). The large central particle had the volume expected of hexameric Orai1 (566 ± 8 nm<sup>3</sup>; Fig. 7 B). A volume distribution of bound peripheral particles for the Orai1-Myc/HA-STIM1 sample had two peaks at 131 ± 2 and 235 ± 4 nm<sup>3</sup> (Fig. 7 C), consistent with the expected volumes of STIM1 monomers and dimers. For the Orai1-Myc/HA-STIM1/ $\sigma$ 1R-FLAG sample, the volume distribution of the peripheral particles had three peaks (62 ± 13, 130 ± 20, and 220 ± 22 nm<sup>3</sup>) corresponding to STIM1 monomers and dimers and a smaller peak consistent with the expected volume of  $\sigma$ 1R monomers (~63 nm<sup>3</sup>; Fig. 7 D). Of the 300 Orai1 complexes analyzed when expressed with STIM1 alone, 73 had bound particles and were either singly or doubly decorated. The total number of bound STIM1 was 96. From the 300 Orai1 complexes analyzed when coexpressed with STIM1 and  $\sigma$ 1R, 76 had bound particles; there were 59 bound STIM1 and 46 bound  $\sigma$ 1R. So the total number of bound STIM1 was reduced by 39% in the presence of  $\sigma$ 1R. AFM images of Orai1 isolated from cells expressing Orai1 and STIM1 revealed, albeit with low frequency, that Orai1 bound to strings of STIM1 (Fig. 7 E). These assemblies were never seen in images from cells coexpressing  $\sigma$ 1R. These results provide evidence for a PM complex of Orai1, STIM1, and  $\sigma$ 1R and for reduced binding of STIM1 to Orai1 in the presence of  $\sigma$ 1R.

### $\sigma$ 1R inhibits SOCE via STIM1 rather than by direct effects on Orai1

Reduced binding of STIM1 to Orai1 caused by  $\sigma$ 1R is expected to reduce SOCE, but  $\sigma$ 1R might also directly inhibit gating of Orai1 channels. To address this possibility, we used the channel-activating domain (CAD) of STIM1, which directly activates Orai1 (Muik et al., 2009; Park et al., 2009; Yuan et al., 2009; Gudlur et al., 2014). mCh-CAD expressed alone in HeLa cells was diffusely distributed in the cytoplasm, but it was peripherally distributed when coexpressed with Orai1 (Fig. 8 A). Addition of extracellular Ca<sup>2+</sup> to HEK or HEK- $\sigma$ 1R cells in Ca<sup>2+</sup>-free HBS had no significant effect on [Ca<sup>2+</sup>]<sub>i</sub>, but there was a substantial increase in [Ca<sup>2+</sup>]<sub>i</sub> in cells expressing CAD, consistent with constitutive activation of SOCE by CAD (Fig. 8, B and C). The response was indistinguishable in HEK and HEK- $\sigma$ 1R cells, suggesting that  $\sigma$ 1R does not directly modulate PM expression of Orai1 nor its activity.

## Discussion

We have shown that  $\sigma$ 1Rs inhibit SOCE by decreasing the effectiveness with which empty stores stimulate Orai1. The target for regulation of SOCE by  $\sigma$ 1R appears to be STIM1 (Fig. 8 D).  $\sigma$ 1R and STIM1 colocalize in the ER; they can be coimmunoprecipitated before and after depletion of Ca<sup>2+</sup> stores, and their interaction is regulated by  $\sigma$ 1R ligands. The agonist, (+) SKF10047, increases binding of STIM1 to  $\sigma$ 1R and further



**Figure 8. Translocation of  $\text{Ca}^{2+}$ -regulating proteins to ER-PM junctions by STIM1.** (A) Confocal images of unstimulated HeLa cells transiently transfected with mCh-CAD alone (left) or with Orai1-CFP (right). Bars, 10  $\mu\text{m}$ . (B) HEK or HEK- $\sigma 1\text{R}$  cells were mock transfected or transfected with CAD, and  $[\text{Ca}^{2+}]_c$  was recorded after addition of 1 mM BAPTA and then restoration of 4 mM extracellular  $\text{Ca}^{2+}$ . WT, wild type. (C) Summary results show peak  $[\text{Ca}^{2+}]_c$  signals evoked by restoration of extracellular  $\text{Ca}^{2+}$  (mean  $\pm$  SD from six replicates). \*\*\*,  $P < 0.001$ , relative to control. Student's  $t$  test. (D) Several proteins, including POST,  $\sigma 1\text{R}$ , and SARAF, associate with STIM1 in ER membranes (Krapivinsky et al., 2011; Palty et al., 2012). Loss of  $\text{Ca}^{2+}$  from the ER causes STIM1 molecules to oligomerize and become trapped, with their cargoes, in ER-PM junctions (green shading) as STIM1 binds to phosphatidylinositol 4,5-bisphosphate and Orai1. STIM1 activates Orai1 and thereby SOCE. The proteins associated with STIM1 also regulate SOCE. SARAF, by competing with STIM1, reduces STIM1 oligomerization and thereby contributes to the termination of SOCE (Palty et al., 2012).  $\sigma 1\text{R}$  competes with STIM1 for binding to Orai1, thereby inhibiting SOCE. POST associates with and inhibits the PM  $\text{Ca}^{2+}$  pump (PMCA) and thereby reduces local  $\text{Ca}^{2+}$  extrusion (Krapivinsky et al., 2011).

inhibits SOCE, whereas the antagonist, BD1047, has the opposite effects. After store depletion,  $\sigma 1\text{R}$  translocates with STIM1 to ER-PM junctions, but  $\sigma 1\text{R}$  slows recruitment of STIM1 and reduces the amount of STIM1 bound to PM Orai1. This reduction in STIM1 binding to Orai1 suggests a likely mechanism for the inhibition of SOCE wherein  $\sigma 1\text{R}$  accompanies STIM1 to ER-PM junctions, where it attenuates the interaction of STIM1

with Orai1. The gap between the ER and PM at the junctions where SOCE occurs is probably too large ( $>9$  nm; Várnai et al., 2007) to be bridged by the short cytosolic loop of  $\sigma 1\text{R}$  (Fig. S1; Hayashi and Su, 2007). The association of  $\sigma 1\text{Rs}$  with PM Orai1 is therefore likely to be mediated by STIM1. Reduced binding of STIM1 to Orai1 in the presence of  $\sigma 1\text{R}$  may be caused by  $\sigma 1\text{R}$  inhibiting the oligomerization of STIM1 or directly reducing the affinity of STIM1 for Orai1.

There are interesting similarities between the behavior of  $\sigma 1\text{Rs}$  and that of other ER membrane proteins, including SARAF (Palty et al., 2012) and POST (partner of STIM1; Krapivinsky et al., 2011). SARAF also translocates to ER-PM junctions in a STIM1-dependent manner, and it promotes deactivation of STIM1 by antagonizing interactions between STIM1 molecules (Palty et al., 2012). Translocation of POST modulates SOCE-evoked  $\text{Ca}^{2+}$  signals because it inhibits the PM  $\text{Ca}^{2+}$  pump that extrudes cytosolic  $\text{Ca}^{2+}$  (Krapivinsky et al., 2011). Hence, after loss of  $\text{Ca}^{2+}$  from the ER, STIM1 both activates SOCE and fine-tunes its activity by delivering additional  $\text{Ca}^{2+}$ -regulating proteins to ER-PM junctions (Fig. 8 D). For  $\sigma 1\text{Rs}$ , the effects of ER luminal  $\text{Ca}^{2+}$  on these delivery mechanisms may operate at two levels. Loss of ER  $\text{Ca}^{2+}$  (or a  $\sigma 1\text{R}$  agonist) releases  $\sigma 1\text{R}$  from its interaction with the ER luminal protein, BiP (Fig. S1; Hayashi and Su, 2007). Store depletion also causes STIM1 to oligomerize and thereby gain affinity for ER-PM junctions. Depletion of  $\text{Ca}^{2+}$  stores may therefore both release  $\sigma 1\text{R}$  from its ER tethers and, via its association with oligomeric STIM1, allow it to accumulate at ER-PM junctions. We focused on SOCE, but recruitment of  $\sigma 1\text{Rs}$  to ER-PM junctions by STIM1 might also be involved in regulation of other PM channels by  $\sigma 1\text{Rs}$  (Maurice and Su, 2009; Su et al., 2010; Kourrich et al., 2013; Pabba, 2013). For example, the L-type  $\text{Ca}^{2+}$  channel is inhibited by  $\sigma 1\text{R}$  (Tchedre et al., 2008) and by depletion of intracellular  $\text{Ca}^{2+}$  stores and STIM1 (Park et al., 2010; Wang et al., 2010). We suggest that STIM1-mediated translocation of  $\sigma 1\text{R}$  to ER-PM junctions may inhibit voltage-gated  $\text{Ca}^{2+}$  entry and may also deliver  $\sigma 1\text{Rs}$  to additional PM targets (Fig. 8 D).

Inhibition of SOCE by  $\sigma 1\text{Rs}$  was invariably accompanied by a decrease in the  $\text{Ca}^{2+}$  content of the ER with no evident change in  $[\text{Ca}^{2+}]_c$ . In contrast, and consistent with a study by López et al. (2012), inhibition of SOCE by expression of Orai1<sup>E106Q</sup> did not consistently affect ER  $\text{Ca}^{2+}$  content: it was normal in HEK cells but reduced in CHO cells. Inhibition of the STIM1-Orai1 interactions that mediate thapsigargin-evoked SOCE are not, therefore, sufficient to explain the effects of  $\sigma 1\text{Rs}$  on ER  $\text{Ca}^{2+}$  content. It may be that  $\sigma 1\text{Rs}$  also interact with STIM2, which plays a major role in maintaining the  $\text{Ca}^{2+}$  content of the stores (Brandman et al., 2007), or with other proteins, such as the sarco/ER  $\text{Ca}^{2+}$  ATPase, as was shown for orosomucoid-like 3 (Cantero-Recasens et al., 2010), or with  $\text{Ca}^{2+}$  channels that mediate  $\text{Ca}^{2+}$  uptake and release from the ER. For example, Sec61 mediates  $\text{Ca}^{2+}$  release from the ER, and it is inhibited by BiP (Schäuble et al., 2012). Expression of  $\sigma 1\text{R}$  might sequester BiP (Fig. S1) and thereby enhance the Sec61-mediated  $\text{Ca}^{2+}$  leak. The decreased  $\text{Ca}^{2+}$  content of the ER might also arise from  $\sigma 1\text{R}$  stabilizing IP<sub>3</sub>R3 and thereby enhancing  $\text{Ca}^{2+}$  transport from the ER to mitochondria (Hayashi and Su, 2007).

The pathophysiological effects  $\sigma 1\text{Rs}$  may, in part, result from inhibition of SOCE and the reduced  $\text{Ca}^{2+}$  content of the ER. The latter may affect protein folding (Hayashi and Su, 2007) and inhibit apoptosis by preventing excessive  $\text{Ca}^{2+}$  transfer to mitochondria (Maurice and Su, 2009; Giorgi et al., 2012).



The effects of  $\sigma 1$ Rs on mitochondrial  $\text{Ca}^{2+}$  uptake are probably finely balanced because  $\sigma 1$ Rs enhance delivery of  $\text{Ca}^{2+}$  to mitochondria at MAMs by stabilizing MAM-associated  $\text{IP}_3$ Rs (Hayashi and Su, 2007), whereas our results show that  $\sigma 1$ Rs reduce the ER  $\text{Ca}^{2+}$  content. The latter could explain the otherwise surprising antiapoptotic effects of  $\sigma 1$ Rs (Wang et al., 2005; Maurice and Su, 2009; Decuypere et al., 2011; Crottès et al., 2013). The  $\sigma 1$ R agonist, cocaine, was recently shown to attenuate SOCE in rat brain microvascular endothelial cells (Brailoiu et al., 2016). The neuroprotective effects of  $\sigma 1$ R agonists after ischemic injury (Katnik et al., 2006) and in patients with amyotrophic lateral sclerosis arising from loss-of-function mutations in  $\sigma 1$ R (Al-Saif et al., 2011; Ono et al., 2014) may also, at least in part, be due to inhibition of SOCE. Hyperactive SOCE may contribute to the motor deficiencies in  $\sigma 1$ R-knock-out mice (Maurice and Su, 2009; Sabino et al., 2009; Mavlyutov et al., 2010) and to neurodegeneration in Alzheimer's (Mishina et al., 2008; Ishikawa and Hashimoto, 2009; Hyrskyluoto et al., 2013) and Parkinson's diseases (Mishina et al., 2005; Hyrskyluoto et al., 2013; Francardo et al., 2014), where expression of  $\sigma 1$ R is reduced. These suggestions prompt consideration of whether  $\sigma 1$ R also interacts with STIM2 because it appears to play the major role in regulating SOCE in central neurons (Berna-Erro et al., 2009).

We conclude that  $\sigma 1$ Rs inhibit SOCE because they associate with STIM1, slow STIM1 recruitment to ER-PM junctions, and reduce its binding to Orai1 after depletion of  $\text{Ca}^{2+}$  stores. Our study highlights a role for STIM1 in translocating  $\sigma 1$ Rs to the PM and establishes  $\sigma 1$ Rs and their ligands as important regulators of SOCE, a ubiquitously expressed  $\text{Ca}^{2+}$  entry pathway (Fig. 8 D).

## Materials and methods

### Materials

(+)-SKF10047 and BD1047 were from Tocris Bioscience. Ionomycin was from MerckEurolab. Thapsigargin was from Alomone Labs. Anti-Myc monoclonal antibody (1:500 dilution for immunoblots; 46-0603), fura 2-AM, and fluo 4-AM were from Thermo Fisher Scientific. Anti-HA (1:500; 16B12) and anti-FLAG (1:500; F3165) monoclonal antibodies were from Covance and Sigma-Aldrich, respectively. The anti- $\sigma 1$ R antibody (1:200; Ab53852), which recognizes a sequence conserved in human and mouse  $\sigma 1$ R, was from Abcam. Custom-made rabbit polyclonal antipeptide antisera to STIM1 (1:100; CDPQHGH GSQRDLTR; the Cys used for conjugation is underlined) and Orai1 (1:200; CEFAWLQDQLDHRGD) were prepared by Sigma-Aldrich. Anti-actin (1:500; A5441) antibody was from Sigma-Aldrich. Anti-mouse (1:1,000) and anti-rabbit (1:1,000) HRP-conjugated secondary antibodies were from Thermo Fisher Scientific and Bio-Rad Laboratories, respectively. Sources of additional materials are provided within the relevant methods.

### Plasmids and siRNA

Plasmids encoding HA-STIM1 and Orai1-Myc/His<sub>6</sub> have been described previously (Willoughby et al., 2012; Balasuriya et al., 2014b). For mCh-STIM1, human STIM1 was subcloned into mCherry-C1 (Takara Bio Inc.) using XbaI and NotI. For  $\sigma 1$ R-FLAG,  $\sigma 1$ R was subcloned into pcDNA3.1/FLAG using HindIII and AgeI. For  $\sigma 1$ R-GFP,  $\sigma 1$ R was subcloned into GFP-N1 (Takara Bio Inc.) using HindIII and KpnI. For  $\sigma 1$ R-V5,  $\sigma 1$ R was subcloned into pcDNA3.1/V5-His-TOPO using HindIII and AgeI. For  $\sigma 1$ R-mKate,  $\sigma 1$ R was subcloned into

mKate2-N (Evrogen) using HindIII and KpnI. The coding sequences of all new constructs were verified. pDsRed2-Mito was from Takara Bio Inc. A pSIREN vector encoding siRNA for  $\sigma 1$ R (5'-GATCCACACGTGGATGGTGGAGTATTCAAGAGATACTCCACCATCCACGTGTTTTTTTGTCTAGCG-3') was used to inhibit expression of  $\sigma 1$ R. pSIREN encoding the luciferase gene was used as a negative control. Both pSIREN constructs were gifts from T.-P. Su (National Institutes of Health, Bethesda, MD; Hayashi and Su, 2004). An expression plasmid (MO70) encoding a dominant-negative form of Orai1 in which Glu-106 is replaced by Gln (Orai1<sup>E106Q</sup>) was a gift from Y. Gwack and S. Srikanth (University of California, Los Angeles, Los Angeles, CA; Srikanth et al., 2012). The expression plasmid for mouse GFP-NFAT1 was a gift from A. Parekh (University of Oxford, Oxford, England, UK; Kar et al., 2011). The mCh-STIM1 CAD expression plasmid was a gift from P. Hogan (La Jolla Institute for Allergy and Immunology, La Jolla, CA; Gudlur et al., 2014), and Orai1-CFP was from D.M.F. Cooper (University of Cambridge, Cambridge, England, UK).

### Cell culture and transfection

All cells were maintained in DMEM with 10% fetal bovine serum and 1% penicillin/streptomycin (Sigma-Aldrich) at 37°C in humidified air with 5% CO<sub>2</sub>. tsA 201 cells were grown to 70% confluence in a 162-cm<sup>2</sup> flask and transfected using calcium phosphate. 50  $\mu$ g of plasmid DNA was mixed with 5 ml of 250 mM CaCl<sub>2</sub> and diluted with 5 ml of medium comprising 275 mM NaCl, 10 mM KCl, 1.4 mM Na<sub>2</sub>HPO<sub>4</sub>, 15 mM glucose, and 42 mM Hepes, pH 7.07. The mixture was added to the cells bathed in 25 ml of fresh growth medium. After 8 h, the medium was replaced with fresh growth medium. Cells were incubated for a further 48 h before being used for experiments.

HEK 293 cells were transfected using polyethylenimine. For cells grown to 70% confluence in 1 well of a 6-well plate, 1  $\mu$ g of plasmid DNA was mixed with 2  $\mu$ l of 7.5 mM polyethylenimine (Polysciences, Inc.) and then diluted with 150  $\mu$ l of serum-free DMEM. The mixture was incubated for 10 min at 20°C and then added to wells containing 2 ml of fresh growth medium for 48 h. The generation of a polyclonal HEK cell line stably expressing mouse  $\sigma 1$ R-V5 (HEK- $\sigma 1$ R cells) was performed as described previously (Xu et al., 2012). These cells were maintained in medium supplemented with 0.8 mg/ml G418 (Thermo Fisher Scientific).

HeLa cells were grown on poly-L-lysine-coated 25-mm glass coverslips and transfected using Lipofectamine 2000 (Thermo Fisher Scientific). For 1 well of a 6-well plate, 2  $\mu$ g of plasmid DNA was diluted in 200  $\mu$ l Opti-MEM and incubated at 20°C for 5 min. This was combined with 200  $\mu$ l Opti-MEM containing 4  $\mu$ l Lipofectamine 2000 and left for a further 20 min at 20°C. The mixture was then added to cells in 2 ml of fresh medium. Cells were incubated for 48 h at 37°C and then used for experiments.

### Measurements of $[\text{Ca}^{2+}]_i$

For measurements of  $[\text{Ca}^{2+}]_i$  in populations of cells, HEK cells were seeded into poly-L-lysine-coated 96-well plates. After 24 h, cells were incubated with 2  $\mu$ M fluo 4-AM in HBS for 60 min at 20°C. HBS had the following composition: 150 mM NaCl, 10 mM KCl, 1 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 10 mM glucose, and 10 mM Hepes, pH 7.3. In  $\text{Ca}^{2+}$ -free HBS,  $\text{Ca}^{2+}$  was omitted, and 1 mM BAPTA was added. For treatments with (+)-SKF10047 and BD1047, 10 mM stock solutions were prepared in DMSO and water, respectively. Cells were pretreated with 25  $\mu$ M (+)-SKF10047 or 10  $\mu$ M BD1047 in serum-free DMEM for 1 h at 37°C before loading cells with fluorescent  $\text{Ca}^{2+}$  indicators. Drug treatments were continued during loading and throughout  $[\text{Ca}^{2+}]_i$  measurements. After loading, cells were washed and incubated in HBS for a further 30 min at 20°C. Fluorescence (excitation at 490 nm and emission at

520 nm) was measured at 20°C using a plate reader that allows on-line additions (FlexStation 3; Molecular Devices). Fluorescence was calibrated to  $[Ca^{2+}]_c$  from

$$[Ca^{2+}]_c = K_D \left( \frac{F - F_{min}}{F_{max} - F} \right),$$

where  $K_D = 345$  nM ( $K_D = 190$  nM at 37°C; Fig. S2 B),  $F$  is the measured fluorescence, and  $F_{max}$  and  $F_{min}$  are the fluorescence values determined after addition of 0.1% Triton X-100 in HBS with 10 mM  $Ca^{2+}$  or 10 mM BAPTA, respectively.

### Measurements of $Mn^{2+}$ entry

Confluent cultures of HEK cells in 96-well plates were loaded with 2  $\mu$ M fura 2-AM using the method described for fluo 4. Fluorescence (excitation at 360 nm and emission at 510 nm) was measured using a plate reader (FlexStation 3) at 1.5-s intervals at 20°C. Quenching of fura 2 fluorescence (which reports unidirectional entry of  $Mn^{2+}$ ) is reported as  $F/F_0$ , where  $F$  is the fluorescence intensity recorded at each time and  $F_0$  is the mean fluorescence intensity measured in the 5 s before addition of  $MnCl_2$ . Monoexponential curve fits to the time course of the changes in  $F/F_0$  were used to compute half-times ( $t_{1/2}$ ) for  $Mn^{2+}$ -evoked fluorescence quenching.

### NFAT translocation assay

HEK cells were seeded onto poly-L-lysine-coated 25-mm coverslips, transfected with GFP-NFAT plasmid using polyethylenimine, and used after 48 h. The distribution of GFP fluorescence was measured before and 40 min after addition of 5  $\mu$ M thapsigargin to cells at 37°C in HBS. Fluorescence (excitation at 488 nm and emission at 510–540 nm) was collected using a confocal microscope (SP5; Leica Biosystems) with an oil-immersion 40 $\times$  objective (NA 1.25). Analyses of nuclear translocation of GFP-NFAT were performed with coded images, which were decoded only when the analysis was complete.

### Analyses of protein expression

Cells were grown in 162-cm<sup>2</sup> flasks. Where appropriate, cells were transfected with 50  $\mu$ g of plasmid DNA using polyethylenimine. Cells were extracted in ice-cold medium (138 mM NaCl, 5 mM KCl, 1 mM  $Na_2HPO_4$ , 7.5 mM glucose, 21 mM Hepes, and 2 mM EDTA, pH 7.4) and centrifuged at 1,000  $g$  for 5 min. Pelleted cells were solubilized at 4°C for 60 min in Triton solution (TS) containing 25 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, and 1 mg/ml protease inhibitor cocktail solution (Roche), pH 7.4, and samples were analyzed by SDS-PAGE followed by immunoblotting.

### Immunoprecipitation analyses

tsA 201 cells, which are SV40-transformed HEK 293 cells, were used because they express heterologous proteins at high levels. Cells were grown in 162-cm<sup>2</sup> flasks and transfected using calcium phosphate. Pretreatments with (+)SKF10047 and BD1047 were for 2 h at 37°C, and stimulation with 5  $\mu$ M thapsigargin was for 30 min at 20°C. Cells were extracted in 25 ml of ice-cold medium, and all subsequent steps were performed at 4°C. The suspension was centrifuged at 1,000  $g$  for 5 min, and pelleted cells were solubilized for 60 min in 500  $\mu$ l TS. After centrifugation (50,000  $g$  for 60 min), 50  $\mu$ l of the supernatant was removed for analysis of total expression (input), and 450  $\mu$ l was incubated with 30  $\mu$ l anti-Myc (EZ View Red) or anti-FLAG beads (Sigma-Aldrich) for 3 h with rotation. Protein-bead complexes were isolated (20,800  $g$  for 10 min) and washed three times in TS, and proteins were eluted either with 50  $\mu$ l of the peptides (1 mg/ml; Sigma-Aldrich), to which the anti-Myc or anti-FLAG antibodies had been raised, or with 50  $\mu$ l Laemmli buffer. The eluted samples were analyzed by SDS-PAGE followed by immunoblotting.

For immunoblots, lanes were loaded with 10  $\mu$ l of the 500- $\mu$ l sample (2% of the entire sample) for the measurement of input and with 10 or 20  $\mu$ l of the 50- $\mu$ l eluate for measurements of immunoprecipitation.

### Isolation of surface biotinylated proteins

tsA 201 cells were grown in 162-cm<sup>2</sup> flasks and transfected using calcium phosphate. After appropriate stimulation, the medium was removed and replaced with 12.5 ml of ice-cold HBS containing 0.2 mg/ml biotin-sulfo-NHS (Thermo Fisher Scientific). After 60 min on ice, cells were washed three times with 15 ml Tris-buffered saline (25 mM Tris-HCl, 150 mM NaCl, and 10 mM EDTA, pH 7.4) and centrifuged at 1,000  $g$  for 5 min, and the pellet was solubilized in 500  $\mu$ l TS for 60 min at 4°C. After centrifugation at 50,000  $g$  for 60 min, the supernatant was incubated with 50  $\mu$ l monomeric avidin-coated agarose beads (Thermo Fisher Scientific) at 4°C for 2 h. Protein-bead complexes were collected at 20,800  $g$  for 10 min, washed three times in TS, and eluted with either 50  $\mu$ l Laemmli buffer for immunoblots or biotin (1 mg/ml in 1 ml TS) for further immunopurification using anti-Myc beads as described in the previous paragraph. For analyses of avidin pull-downs of biotinylated proteins (Fig. 5, C–E), 2% of the total sample was loaded as input, and 40% of the Laemmli sample was loaded in the surface biotinylation lanes.

### Immunostaining

HeLa cells were seeded on poly-L-lysine-coated glass coverslips, transfected, and used after 48 h. After stimulation, cells were washed with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 10 mM  $Na_2HPO_4$ , and 2 mM  $KH_2PO_4$ ), fixed with 4% paraformaldehyde for 20 min, and permeabilized with 0.5 mg/ml saponin for 60 min (Sigma-Aldrich) in blocking solution (5% goat serum and 3% BSA in PBS). Cells were stained with primary antibody in blocking solution (PBS containing 3% BSA and 5% goat serum) for 60 min at 20°C, washed twice with PBS, and then incubated in the dark with secondary antibody in blocking solution for 60 min at 20°C, washed with PBS, dried, mounted onto a glass microscope slide, and stored at 4°C. Cells were imaged using an oil-immersion 60 $\times$  objective (NA 1.40) using a confocal microscope (SP5; Leica Biosystems). For both Pearson's and Mander's coefficient measurements, images were analyzed with ImageJ (National Institutes of Health) using the JACoP plugin. For Mander's coefficient, only pixels in which HA-STIM1 (or  $\sigma$ 1R-EGFP) was detected were considered, and the fraction of those pixels in which Orai1-Myc was also detected was then computed to provide the colocalization coefficient.

### TIRF microscopy

Coverslips were mounted on a TIRF microscope (IX51 inverted microscope [Olympus] with a 100 $\times$  oil-immersion objective [NA 1.49] coupled to an electron-multiplying charged-coupled device camera [iXon; Andor Technology] and 488-nm argon ion and 561-nm diode lasers). Cells were incubated with HBS at 20°C and imaged (1 image/s) by exciting  $\sigma$ 1R-GFP at 488 nm (emission at 510–540 nm) and mCh-STIM1 at 561 nm (emission at 610–650 nm). For each experiment, there were suitable controls, with cells expressing the EGFP-tagged protein alone and the mCherry/mKate-tagged protein alone to ensure there was no bleed through. For depletion of stores, cells were incubated with 1  $\mu$ M thapsigargin in  $Ca^{2+}$ -free HBS. Fluorescence intensities were quantified using the time series analyzer plugin V2.0 in ImageJ. Individual regions of interest within the cell were selected, and the data were analyzed as  $F/F_0$ , where  $F$  and  $F_0$  are the fluorescence intensities at each time and at the start of the experiment, respectively.

### AFM

tsA 201 cells expressing appropriate combinations of Orai1-Myc-His,  $\sigma$ 1R-FLAG, and HA-STIM1 were treated with thapsigargin, followed

by biotin-sulfo-NHS, and then purified using sequential avidin and anti-Myc affinity chromatography, as described in the Isolation of surface biotinylated proteins section. About 45  $\mu$ l of proteins was added to a 1-cm<sup>2</sup> mica disk, incubated at 20°C for 10 min, gently washed with water, and dried under nitrogen. Samples were imaged in air using an atomic force microscope (Multimode; Bruker). The silicon cantilever (OTESPA; Bruker) was set at a drive frequency of 271–321 kHz and spring constant of 12–103 N/m. The scan rate was 3 Hz, and the applied imaging force was kept as low as possible (target amplitude of 1.0 V and amplitude set point of 0.7–1.0 V). Molecular volumes for individual particles were determined using an image processor (version 5; Scanning Probe). For particles within complexes, particle heights (h) and radii (r) were measured manually using Nanoscope software. Particle volumes ( $V_m$ ) were then calculated from

$$V_m = \frac{\pi h(3r^2 + h^2)}{6}.$$

Molecular volume ( $V_c$ ), based on a known molecular mass ( $M_0$ ), was calculated from

$$V_c = \frac{M_0(V_1 + dV_2)}{N_0},$$

where  $N_0$  is Avogadro's number,  $V_1$  is the specific particle volume (0.74 cm<sup>3</sup>/g),  $V_2$  is the water specific volume (1 cm<sup>3</sup>/g), and d is the extent of hydration (assumed to be 0.4 g H<sub>2</sub>O/g protein).

### Data analysis

Most results are presented as mean  $\pm$  SEM from  $n$  independent experiments. Statistical analysis used Student's  $t$  test or analysis of variance (ANOVA) followed by Tukey's posthoc test as appropriate.

### Online supplemental material

Fig. S1 illustrates key features of  $\sigma$ 1R, and Table S1 describes ligands targeting  $\sigma$ 1R. Fig. S2 shows the effects of expressing  $\sigma$ 1Rs in HEK cells on SOCE and the Ca<sup>2+</sup> content of the intracellular stores. Fig. S3 shows the effects of  $\sigma$ 1R ligands on SOCE in CHO and HEK cells. Fig. S4 shows the effects of  $\sigma$ 1R ligands on SOCE and the Ca<sup>2+</sup> content of the intracellular stores in MDA-MB-231 human breast cancer cells. Fig. S5 shows the effects of  $\sigma$ 1R ligands on SOCE in HEK- $\sigma$ 1R cells overexpressing STIM1 and Orai1. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.201506022/DC1>.

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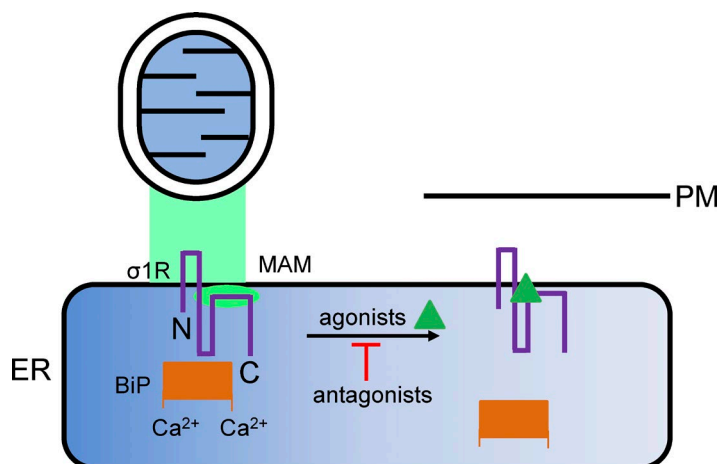
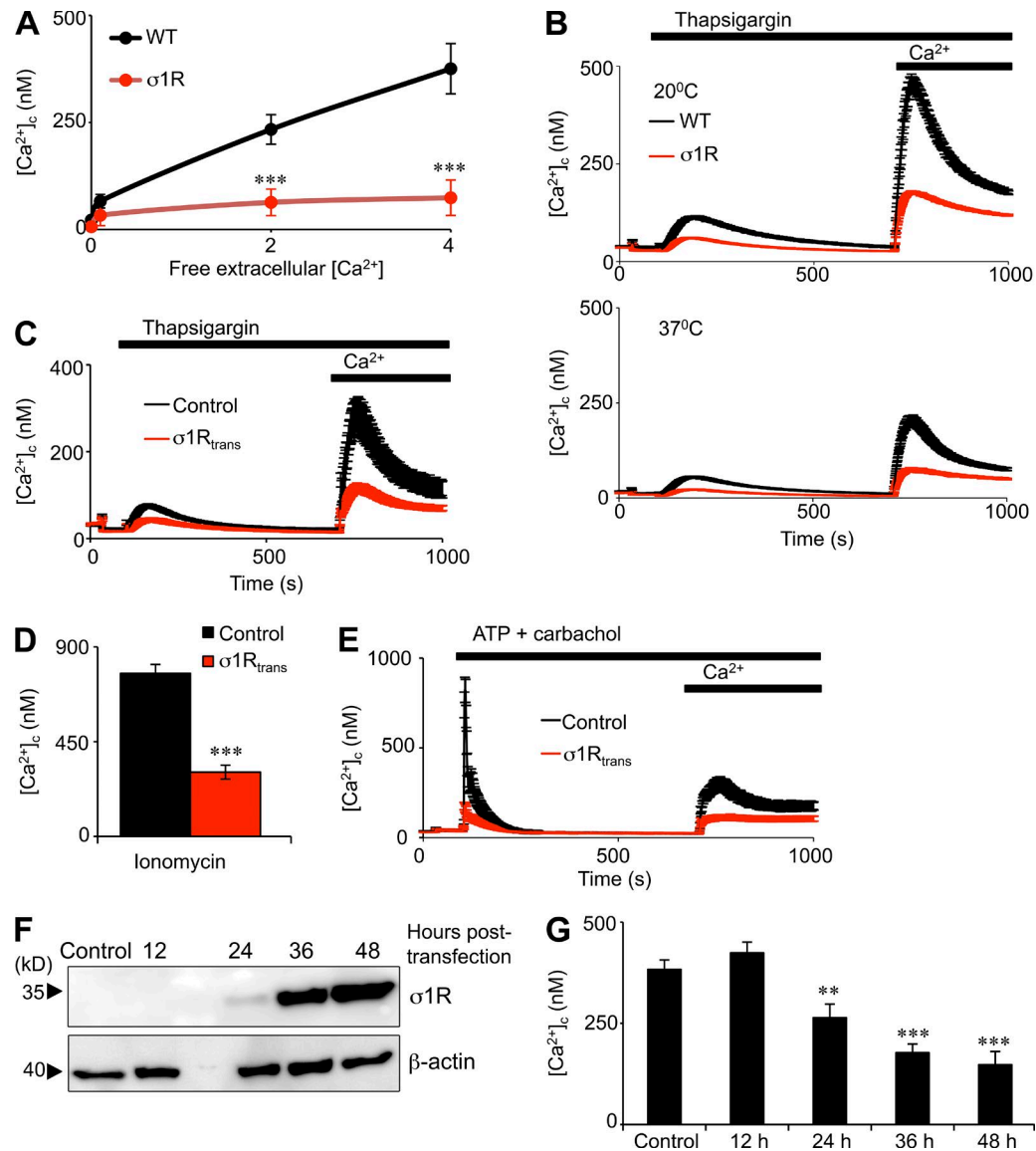
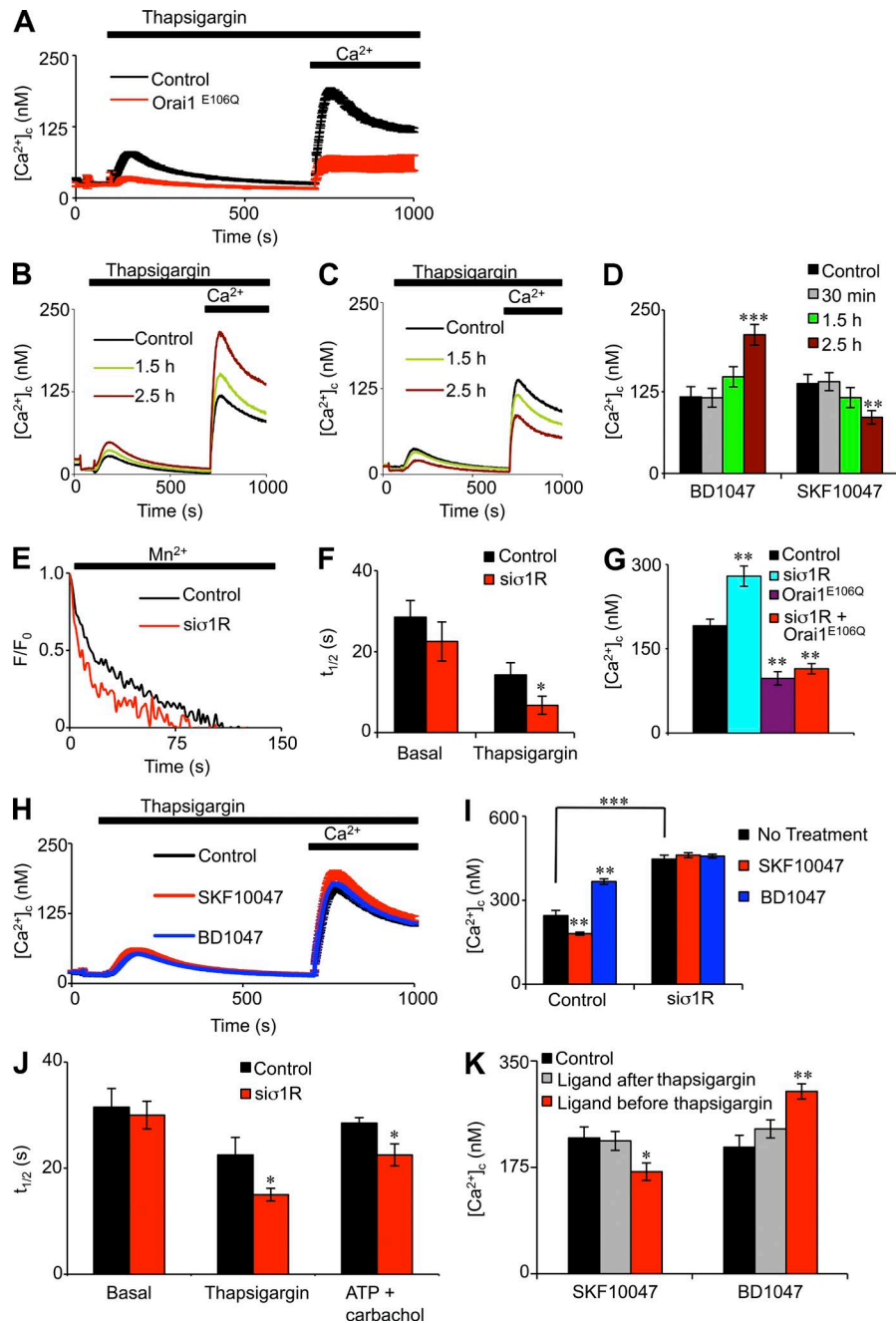


Figure S1. **Key features of  $\sigma 1R$ .**  $\sigma 1R$  comprises 223 residues. It is expressed predominantly in ER membranes, where it adopts the topology shown with two transmembrane domains, a short cytosolic loop, and luminal N and C termini. The N terminus includes an ER retention signal. Residues important for ligand recognition are shown by the green oval.  $\sigma 1Rs$  reside, along with some  $IP_3Rs$ , within MAMs, regions of close contact between the ER and mitochondria that allow  $Ca^{2+}$  and lipid exchanges between them (Hayashi and Su, 2004, 2007).  $IP_3Rs$  associated with  $\sigma 1Rs$  in MAMs are protected from degradation and can thereby deliver sustained physiological  $Ca^{2+}$  signals to mitochondria (Hayashi and Su, 2007). Within MAMs,  $\sigma 1Rs$  also associate with the luminal  $Ca^{2+}$ -binding protein and chaperone, BiP. The interaction between  $Ca^{2+}$ -BiP and  $\sigma 1R$  is disrupted by loss of  $Ca^{2+}$  from the ER or by  $\sigma 1R$  agonists. This frees  $\sigma 1Rs$  to move within ER membranes and establish contacts with different membranes, notably the PM (Hayashi and Su, 2007). The present study demonstrates that STIM1 can then deliver  $\sigma 1Rs$  to specific PM domains.





**Figure S2.  $\sigma$ 1Rs inhibit SOCE-mediated  $\text{Ca}^{2+}$  signals.** (A) Experiments similar to those shown in Fig. 1 D were used to measure thapsigargin-evoked SOCE after restoration of different extracellular  $\text{Ca}^{2+}$  concentrations to wild-type (WT) HEK or HEK- $\sigma$ 1R cells. Results show peak increases in  $[\text{Ca}^{2+}]_i$  after restoration of extracellular  $\text{Ca}^{2+}$  (concentrations refer to the free  $[\text{Ca}^{2+}]$ ; mean  $\pm$  SEM;  $n = 3$  with six replicates in each experiment). (B)  $\text{Ca}^{2+}$  signals evoked by thapsigargin and then restoration of extracellular  $\text{Ca}^{2+}$  at 20°C or 37°C. Results show mean  $\pm$  SD from six replicates. (C) Populations of fluo 4-loaded HEK cells transiently expressing  $\sigma$ 1R-V5 ( $\sigma$ 1R<sub>trans</sub>) or mock transfected (control) were stimulated with 5  $\mu\text{M}$  thapsigargin in  $\text{Ca}^{2+}$ -free HBS before addition of 4 mM extracellular  $\text{Ca}^{2+}$ . Results show the mean  $\pm$  SD of six replicates. (D) Experiments similar to those shown in Fig. 1 (G and H) were used to assess the size of the intracellular  $\text{Ca}^{2+}$  stores in HEK cells transiently expressing  $\sigma$ 1R-V5. 5  $\mu\text{M}$  ionomycin was added to cells in  $\text{Ca}^{2+}$ -free HBS, and the peak increases in  $[\text{Ca}^{2+}]_i$  were recorded (mean  $\pm$  SD from six replicates). (E) Populations of fluo 4-loaded HEK- $\sigma$ 1R<sub>trans</sub> or mock-transfected (control) cells were stimulated with 100  $\mu\text{M}$  ATP and 100  $\mu\text{M}$  carbachol in  $\text{Ca}^{2+}$ -free HBS before addition of 4 mM extracellular  $\text{Ca}^{2+}$ . Results show mean of six replicates. (F) Immunoblot showing  $\sigma$ 1R expression at the indicated times after transfection. (G) Peak increases in  $[\text{Ca}^{2+}]_i$  evoked by SOCE recorded at the indicated times after transient transfection of HEK cells with  $\sigma$ 1R. Results show mean  $\pm$  SD from six replicates. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ , relative to control (Student's  $t$  test in A and D and ANOVA followed by Tukey's posthoc analysis in G). The results show that transient expression of  $\sigma$ 1Rs has similar effects to stable expression, namely inhibition of SOCE and a reduction in the  $\text{Ca}^{2+}$  content of the intracellular stores.



**Figure S3. Time course of the effects of  $\sigma$ 1R ligands on SOCE.** (A) Populations of fluo 4-loaded CHO cells transiently transfected with Orai1<sup>E106Q</sup> or mock transfected were stimulated with 5  $\mu$ M thapsigargin in Ca<sup>2+</sup>-free HBS before restoration of 4 mM extracellular Ca<sup>2+</sup>. Results show mean  $\pm$  SD of six replicates. (B and C) HEK- $\sigma$ 1R cells were pretreated with 10  $\mu$ M BD1047 (B) or 25  $\mu$ M (+)SKF10047 (C) for the indicated times, with the final 1.5 h at 20°C (during dye loading) and the preceding 1 h (for the 2.5-h incubation) at 37°C. Ca<sup>2+</sup> signals evoked by thapsigargin in Ca<sup>2+</sup>-free HBS and then restoration of extracellular Ca<sup>2+</sup> were recorded after the preincubations. (D) Summary results show peak increases in [Ca<sup>2+</sup>]<sub>i</sub> evoked by SOCE. (E) Experiments similar to those shown in Fig. 2 C were used to measure quenching of fura 2 fluorescence in CHO cells treated with siRNA to  $\sigma$ 1R or with control plasmid. Fura 2-loaded cells were treated with thapsigargin (5  $\mu$ M for 10 min) in nominally Ca<sup>2+</sup>-free HBS before addition of 5 mM MnCl<sub>2</sub>. Results show normalized fluorescence intensity (F/F<sub>0</sub>) for six replicates. (F) Summary results show half-times (t<sub>1/2</sub>) for fluorescence quenching before (basal) and after thapsigargin treatment. The results demonstrate that loss of  $\sigma$ 1Rs in CHO cells increases unidirectional entry of Mn<sup>2+</sup> through the SOCE pathway. (G) Summary results show peak increases in [Ca<sup>2+</sup>]<sub>i</sub> evoked by SOCE after thapsigargin treatment (1  $\mu$ M) in CHO cells transfected with siσ1R, Orai1<sup>E106Q</sup>, or both. (H) Typical traces show the effects of pretreatment with ligands of  $\sigma$ 1R (+)SKF10047, 25  $\mu$ M; BD1047, 10  $\mu$ M) on thapsigargin-evoked Ca<sup>2+</sup> signals in HEK- $\sigma$ 1R cells treated with siRNA to  $\sigma$ 1R. Fluo 4-loaded cells were stimulated with 5  $\mu$ M thapsigargin in Ca<sup>2+</sup>-free HBS before restoration of 4 mM extracellular Ca<sup>2+</sup>. (I) Summary results show peak increases in [Ca<sup>2+</sup>]<sub>i</sub> evoked by 5  $\mu$ M ionomycin. (J) Summary results show half-times (t<sub>1/2</sub>) for fluorescence quenching before (basal) and after treatment with thapsigargin (5  $\mu$ M for 10 min) or carbachol with ATP (100  $\mu$ M of each for 3.5 min). The results demonstrate that the effects of  $\sigma$ 1R ligands on thapsigargin-evoked Ca<sup>2+</sup> release and SOCE are abolished after treatment with siRNA for  $\sigma$ 1R. (K) Fluo 4-loaded HEK- $\sigma$ 1R cells were incubated with 25  $\mu$ M (+)SKF10047 or 10  $\mu$ M BD1047 for 2 h in Ca<sup>2+</sup>-free HBS at 20°C. Thapsigargin (1  $\mu$ M for 10 min) was added before or after addition of the  $\sigma$ 1R ligands, and the increase in [Ca<sup>2+</sup>]<sub>i</sub> evoked by SOCE was measured after restoration of extracellular Ca<sup>2+</sup>. The results show that the ligands have less effect on SOCE when added after store depletion. \*, P < 0.05; \*\*, P < 0.01; \*\*\*, P < 0.001, relative to control (ANOVA followed by Tukey's posthoc analysis for D, G, and I-K; Student's t test for F and for comparison of no treatment conditions in I). (D and F-K) Results are mean  $\pm$  SEM (n = 3).

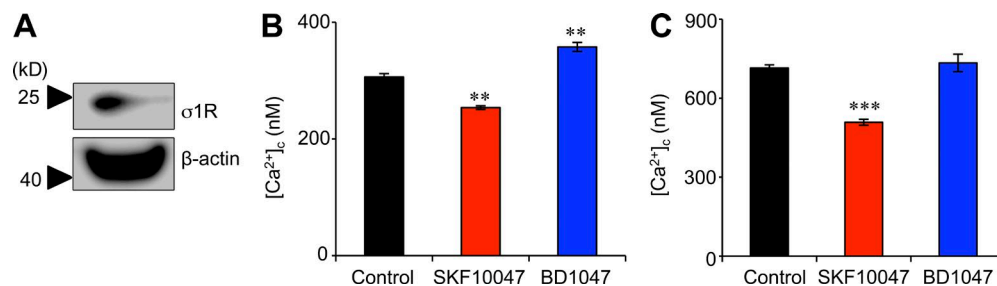


Figure S4. **Effects of ligands of  $\sigma$ 1R on SOCE and intracellular  $\text{Ca}^{2+}$  stores in MDA-MB-231 breast cancer cells.** (A) Immunoblot showing detection of  $\sigma$ 1R in a lysate of MDA-MB-231 cells. (B) Populations of fluo 4-loaded MDA-MB-231 cells were pretreated with 25  $\mu\text{M}$  (+)SKF10047 or 10  $\mu\text{M}$  BD1047 before addition of 5  $\mu\text{M}$  thapsigargin in  $\text{Ca}^{2+}$ -free HBS and then measurement of SOCE after restoration of 4 mM extracellular  $\text{Ca}^{2+}$  after 10 min. Results show the peak  $[\text{Ca}^{2+}]_i$  evoked by the addition of extracellular  $\text{Ca}^{2+}$ . (C) The  $\text{Ca}^{2+}$  content of the intracellular stores was assessed after the same pretreatments with  $\sigma$ 1R ligands by addition of 5  $\mu\text{M}$  ionomycin in  $\text{Ca}^{2+}$ -free HBS. Results show the peak increases in  $[\text{Ca}^{2+}]_i$  evoked by ionomycin. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ , relative to control treatment (ANOVA followed by Tukey's posthoc analysis). Results are mean  $\pm$  SEM ( $n = 3$ ).

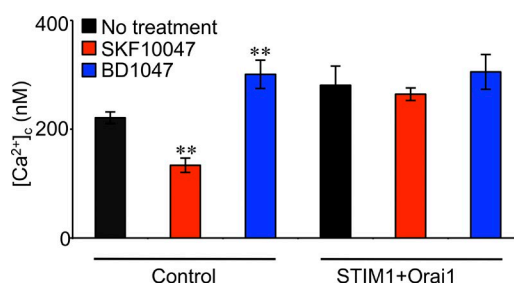


Figure S5. **Effects of ligands of  $\sigma$ 1R on SOCE in cells overexpressing STIM1 and Orai1.** Populations of fluo 4-loaded HEK- $\sigma$ 1R cells were either mock transfected (control) or transfected with STIM1 and Orai1 before pretreatment with 25  $\mu\text{M}$  (+)SKF10047 or 10  $\mu\text{M}$  BD1047. SOCE was then measured by addition of extracellular  $\text{Ca}^{2+}$  to cells treated with thapsigargin in  $\text{Ca}^{2+}$ -free HBS. Results (mean  $\pm$  SD; six replicates) show the peak  $[\text{Ca}^{2+}]_i$  evoked by addition of extracellular  $\text{Ca}^{2+}$ . \*\*,  $P < 0.01$ , relative to no treatment, using ANOVA followed by Tukey's posthoc analysis.



Table S1. **Ligands targeting  $\sigma$ 1R**

Antagonists	Agonists
<b>BD1047</b>	Cocaine
Haloperidol	Dimemorfan
Metaphit	Donepezil
NE100	DTG
Rimcazole	Fluvoxamine
	Ibogaine
	Methamphetamine
	<i>N,N</i> -dimethyltryptamine (DMT)
	Panamesine
	PPBP
	PPCC
<b>Selective for <math>\sigma</math>1R versus <math>\sigma</math>2R</b>	Igmesine
	MR22
	(+)-Pentazocine
	PRE-084
	SA4503
	<b>(+)-SKF10047</b>
<b>Endogenous neurosteroids</b>	
Progesterone	Dehydroepiandrosterone (DHEA)
	Pregnenolone

This list is incomplete, but it illustrates the diversity of drugs that interact with  $\sigma$ 1Rs, the overlap with drugs that are important in the clinic or as drugs of abuse, and drugs used in the present study (bold). The  $\sigma$ 1R is probably unrelated to  $\sigma$ 2R, although the two have overlapping pharmacology. Drug actions: cocaine, inhibitor of catecholamine uptake (Schwartz et al., 2010) and drug of abuse. Dimemorfan, centrally acting cough suppressant (Shin et al., 2005). Donepezil (Aricept), reversible inhibitor of acetylcholinesterase used to treat Alzheimer's disease (Ramakrishnan et al., 2014). DMT, hallucinogen and possible endogenous ligand of  $\sigma$ 1Rs (Fontanilla et al., 2009). Fluvoxamine, selective serotonin reuptake inhibitor (SSRI) used to treat depression (Hindmarch and Hashimoto, 2010). Many other SSRIs are also agonists of  $\sigma$ 1Rs. Haloperidol (Dozic and Serenace), dopamine D2 receptor antagonist used to treat schizophrenia and other psychotic disorders (Maurice and Su, 2009). Ibogaine, psychoactive natural product. Potential utility in treatment of drug craving (Mach et al., 1995). Igmesine, possible utility in treatment of depression (Kulkarni and Dhir, 2009). Methamphetamine (crystal meth and ice), stimulates release of catecholamines and blocks their reuptake (Nguyen et al., 2005) and is a drug of abuse. Panamesine, possible utility in treatment of schizophrenia (Kulkarni and Dhir, 2009). Pentazocine, opioid analgesic. Closely related to SKF10047 (Hayashi and Su, 2005). Rimcazole, adverse effects prevented use as a potential anticancer therapy (Happy et al., 2015). BB1047, *N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2,2-(dimethylamino)ethylamine; DTG, 1,3-di-*o*-tolylguanidone; Igmesine, (*R*)-(+)-*N*-cyclopropylmethyl- $\alpha$ -ethyl-*N*-methyl- $\alpha$ -[2E]-3-phenyl-2-propenyl]benzenemethanamine hydrochloride; MR22, (-)-methyl (1*S*,2*R*)-2-[[1-adamantyl(methylamino)methyl]-1-phenylcyclopropanecarboxylate; NE100, 4-methoxy-3-(2-phenylethoxy)-*N,N*-dipropylbenzeneethanamine hydrochloride; PPBP, 4-phenyl-1-(4-phenylbutyl)-piperidine maleate; PPCC, (*S*\*,*R*\*)-2-[[4-hydroxy-4-phenyl-1-piperidinyl)methyl]-1-[4-methylphenyl]-cyclopropanecarboxylic acid methyl ester; PRE-084, 2-[4-morpholinethyl] 1-phenylcyclohexanecarboxylate hydrochloride; SA 4503, 1-[2-[3,4-dimethoxyphenyl]ethyl]-4-[3-phenylpropyl]-piperazine dihydrochloride; (+)-SKF10047, [2*S*-(2 $\alpha$ ,6 $\alpha$ ,11*R*\*)]-1,2,3,4,5,6-hexahydro-6,11-dimethyl-3-(2-propenyl)-2,6-methano-3-benzazocin-8-ol hydrochloride.

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